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<p>(51) International Patent Classification ⁶ : A01H 5/00, C07K 14/415, 16/16, C12N 1/13, 1/21, 5/10, 15/29, 15/64, 15/82</p>	<p>A1</p>	<p>(11) International Publication Number: WO 98/21938 (43) International Publication Date: 28 May 1998 (28.05.98)</p>
<p>(21) International Application Number: PCT/US97/21336 (22) International Filing Date: 18 November 1997 (18.11.97) (30) Priority Data: 60/031,040 18 November 1996 (18.11.96) US 60/061,328 8 October 1997 (08.10.97) US (71) Applicant: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US). (72) Inventors: REA, Philip, A.; 2941 Berkley Road, Ardmore, PA 19003 (US). LU, Yu-Ping; 2403 Manor Avenue, Havertown, PA 19083 (US). LI, Ze-Sheng; Apartment 3A, 211 Lazaretto Road, Prospect Park, PA 19076 (US). (74) Agents: LEARY, Kathryn et al.; Panitch Schwarze Jacobs & Nadel, P.C., One Commerce Square, 22nd floor, 2005 Market Street, Philadelphia, PA 19103-7086 (US).</p>		<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.</p>
<p>(54) Title: GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS (57) Abstract The invention includes an isolated DNA encoding a plant GS-X pump polypeptide and an isolated preparation of a plant GS-X pump polypeptide. Also included is an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS-X pump gene. The invention also includes cells, vectors and transgenic plants having an isolated DNA encoding a plant GS-X pump and methods of use thereof. In addition, the invention relates to plant GS-X pump promoter sequences and the uses thereof.</p>		

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GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS

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BACKGROUND OF THE INVENTION

Animal and plant cells have the capacity to eliminate a diversity of lipophilic toxins from the cytosol following conjugation of the toxin with glutathione (GSH) (Ishikawa et al., 1997, *Bioscience Reports*. 17:189-208; Martinoia et al., 1993, *Nature* 364:247-249; Li et al., 1995, *Plant Physiol.* 107:1257-1268). This process is mediated by the glutathione S-conjugate (GS-X) pumps which are novel MgATP-dependent transporters that catalyze the efflux of GS-conjugates and glutathione disulfide (GSSG) from the cytosol via the plasma membrane and/or endomembranes. GS-X pumps are considered to constitute a terminal phase of xenobiotic detoxification in animals and plants.

The metabolism and detoxification of xenobiotics comprises three main phases (Ishikawa, 1992, *supra*). Phase I is a preparatory step in which toxins are oxidized, reduced or hydrolyzed to introduce or expose functional groups having an appropriate reactivity. Cytochrome P450 monooxygenases and mixed function oxidases are examples of phase I enzymes. In phase II, the activated derivative is

conjugated with GSH, glucuronic acid or glucose. In the case of the GSH-dependent pathway, S-conjugates of GSH are formed by cytosolic glutathione-S-transferases (GSTs). In the final phase, phase III, of the GSH-dependent pathway, GS-conjugates are eliminated from the cytosol by the GS-X pump.

5 The GS-X pump is unique in its exclusive use of MgATP, rather than preformed transmembrane ion gradients, as a direct energy source for organic solute transport. Although an understanding of the constituents of GS-X pumps is relevant to an understanding of the mechanism by which cells combat, for example, chemotherapeutic agents and herbicides, there has until recently been a paucity of
10 information on the molecular identity of GS-X pumps, particularly in plants.

A 190 kDa membrane glycoprotein encoded by the human multidrug resistance-associated protein gene (*MRP1*) has been implicated in the resistance of small cell lung cancer cell lines to a number of chemotherapeutic drugs (Cole *et al.*, 1992, *Science* 258:1650-1654). This glycoprotein catalyzes the MgATP-dependent
15 transport of leukotriene C₄ and related glutathione-S-conjugates (Leier *et al.*, 1994, *J. Biol. Chem.* 269:27807-27810; Muller *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:13033-13037; Zamam *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92:7690-7694).

MRP1 is a member of the ATP binding cassette (ABC) superfamily of transporter proteins. Distributed throughout the major taxa, ABC transporters catalyze
20 the MgATP-dependent transport of peptides, sugars, ions and lipophiles across membranes. ABC transporters comprise one or two copies each of two basic structural elements, a hydrophobic integral membrane sector containing approximately six transmembrane α helices and a cytoplasmically oriented ATP-binding domain known as a nucleotide binding fold (NBF) (Hyde *et al.*, 1990, *Nature* 346:362-365; Higgins, 1995, *Cell* 82:693-696). The NBFs are a diagnostic feature of ABC transporters and
25 are 30% identical between family members over a span of about 200 amino acid residues, having two regions known as a Walker A and a Walker B box (Walker *et al.*, 1992, *EMBO J.* 11:945-951), and also having an ABC signature motif (Higgins, 1995, *supra*).

ABC family members in eukaryotes include mammalian P-glycoproteins (P-gps or MDRs), some of which are implicated in drug resistance and others in lipid translocation (Ruetz *et al.*, 1994, *Cell* 77:1071-1081), the pleiotropic drug resistance protein (PDR5) and STE6 peptide mating pheromone transporter of yeast, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel, the malarial *Plasmodium falciparum* chloroquine transporter (PFMDR1) and the major histocompatibility (MHC) transporters responsible for peptide translocation and antigen presentation (Balzi *et al.*, 1994, *J. Bioenerg. Biomemb.* 27:71-76; Higgins, 1995, *supra*).

Sequence comparisons between MRP1 and other ABC transporters reveal two major subgroups among these proteins (Cole *et al.*, 1992, *supra*; Szczyepka *et al.*, 1994, *J. Biol. Chem.* 269:22853-22857). One subgroup comprises MRP1, the *Saccharomyces cerevisiae* cadmium factor (*YCF1*) gene, the *Leishmania* P-glycoprotein-related molecule (*Lei/PgpA*) and the CFTRs. The other subgroup comprises the multiple drug resistance proteins (MDRs), MHC transporters and STE6.

The invention described herein relates to bioremediation (specifically phytoremediation), plant responses to herbicides, plant-pathogen interactions and plant pigmentation.

With respect to bioremediation, the massive global expansion in industrial and mining activities during the last two decades together with changes in agricultural practices, has markedly increased contamination of groundwaters and soils with heavy metals. Indeed, it is estimated that the annual toxicity of metal emissions exceeds that of organics and radionuclides combined (Nriagu *et al.*, 1988, *Nature* 333:1340138). Since soil and water contamination results in the uptake of heavy metals and toxins by crop plants, and eventually humans, there remains a need for a means of manipulating the ability of a plant to sequester compounds from the soil in order to better manage soil detoxification through bioremediation using native species or genetically engineered organisms.

Regarding herbicides, these compounds are generally low molecular weight, lipophilic compounds that readily penetrate cells in a passive manner. Having entered cells, herbicides inhibit plant-specific processes such as photosynthetic electron transport (e.g., atrazine, chlortoluron) or the biosynthesis of essential amino acids (e.g., glyphosate, chlorsulfuron or phosphotricine), porphyrins (e.g., acido-fluorfen), carotenoids (e.g., norflurazon), fatty acids (e.g., diclofop) or cellulose (e.g., dichlobenil) (Boger *et al.*, 1989, *Target Sites of Herbicide Action*, CRC Press, Boca Raton, FL; Devine *et al.*, 1993, *Physiology of Herbicide Action*, Prentice Hall, Englewood Cliffs, NJ). Plants that are naturally tolerant of certain herbicides either contain a cellular target that does not interact with the herbicide, have efficient systems for inactivation of the herbicide, or have a high capacity for excluding or eliminating the herbicide from the target.

Herbicide metabolism comprises the three phases described above for general xenobiotic metabolism. The first two phases (the first being oxidation and hydrolysis and the second being conjugation with GSH or glucose) contribute to detoxification by decreasing the intrinsic biochemical activity of the herbicide and/or by increasing its hydrophilicity. These two phases render the herbicide less mobile in the plant. The third phase (compartmentation) is often critical for sustained detoxification since the conjugates themselves may interfere with metabolism. For example, the herbicide synergist tridiphane, is converted to its corresponding GS-conjugate in plants to generate a potent inhibitor of atrazine metabolism. (Lamoureux *et al.*, 1986, *Pestic. Biochem. Physiol.* 26:323-342).

Likewise, and more generally, GS-conjugates of any given herbicide would be expected to act as end-product inhibitors of GSTs and thereby impair long-term detoxification unless they are removed from the intracellular compartment, usually the cytosol, in which they are formed. Since the vacuolar GS-X pump of plants is known to transport several GS-herbicide conjugates, for example, those of the chloroacetanilide herbicides (metolachlor) and triazines (simetryn) (Martinoia *et al.*, 1993, *supra*; Li *et al.*, 1995, *supra*), there is a long felt need for a knowledge of the

molecular identity of this transporter or family of transporters. Such knowledge will enable the development of new strategies for increasing or decreasing the resistance of plants to herbicides.

5 With regard to plant-pathogen interactions, a key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. The majority of the research on plant-pathogen interactions has centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of
10 intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, the discovery of the molecular mechanism by which these compounds are sequestered within a plant is crucial to the development of plants with increased pathogen resistance.

15 With regard to plant pigmentation, functional analyses of the maize gene, *Bronze-2*, which participates in anthocyanin pigment biosynthesis, suggest that one of the endogenous substrates for the plant vacuolar GS-X pump are anthocyanin-GS conjugates (Marrs *et al.*, 1995, *Nature*, 375:397-400). Anthocyanins share a common biosynthetic origin and core structure based on cyanidin-3-glucoside. It is through the species-specific decoration of cyanidin-3-glucoside by hydroxylation,
20 methylation, glucosylation and acylation that the wide spectrum of red, blue and purple colors in the vacuoles of flowers, fruits and leaves is produced. The molecular nature of the plant GS-X pump which mediates transport of anthocyanin-GS conjugates was not known in the art until the present invention. There remains a need to determine the molecular nature of the GS-X pump responsible for transport of anthocyanin-GS
25 conjugates in order that plant coloration may be manipulated at the molecular level.

The present invention satisfies the aforementioned needs.

BRIEF SUMMARY OF THE INVENTION

The invention includes an isolated DNA encoding a plant GS-X pump polypeptide. In one aspect, the isolated DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.

The invention also includes an isolated preparation of a polypeptide comprising a plant GS-X pump. In one aspect of this aspect of the invention, the polypeptide is selected from the group consisting of *AtMRP1*, *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof having GS-X pump activity.

Also included in the invention is a recombinant cell comprising an isolated DNA encoding a plant GS-X pump polypeptide. In one aspect, the cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

Further included in the invention is a vector comprising an isolated DNA encoding a plant GS-X pump polypeptide.

The invention also includes an antibody specific for a plant GS-X pump polypeptide.

In addition, an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene is included in the invention and a cell and a vector comprising this isolated preparation of a nucleic acid are further included.

The invention also relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-X pump.

In addition, the invention relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.

Further, there is included a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.

The invention further relates to an isolated DNA comprising a plant GS-X pump promoter sequence. In one aspect, the promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.

Also included in this aspect of the invention is a cell and a vector
5 comprising an isolated DNA comprising a plant GS-X plant promoter sequence.

The invention additionally relates to a transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising a GS-X pump promoter sequence, wherein the GS-X pump promoter sequence is selected from the group consisting of an *AtMRP1*, an *AtMRP2* and a *YCF1* promoter sequence.
10 The promoter sequence may also have operably fused thereto a reporter gene.

There is also included in the invention a method of identifying a compound capable of affecting the expression of a plant GS-X gene. The method comprises providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto, adding to the
15 cell a test compound, and measuring the level of reporter gene activity in the cell, wherein a higher or a lower level of reporter gene activity in the cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that the test compound is capable of affecting the expression of a plant GS-X pump gene.

20 In addition, the invention relates to a method of removing xenobiotic toxins from soil. The method comprises growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

Also included is a method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA
25 encoding a GS-X pump.

The invention further relates to a method of generating a transgenic pathogen resistant plant comprising introducing to the cells of the plant an isolated DNA encoding a GS-X pump, wherein the pump is capable of transporting glutathionated isoflavonoid alexins into the cells of the plant.

Additionally, there is included a method of manipulating plant pigmentation comprising modulating the expression of a GS-X pump protein in the plant, wherein the GS-X pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

5 The invention also relates to a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-X pump, wherein the DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

10 Further included is a method of manipulating the expression of a gene in a plant cell. The method comprises operably fusing a GS-X pump promoter sequence to the DNA sequence encoding the gene to form a chimeric DNA, and generating a transgenic plant, the cells of which comprise the chimeric DNA, wherein upon activation of the GS-X pump promoter sequence, the expression of the gene is manipulated.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of graphs depicting differential sensitivities of DYT165 cells (wild type, Figure 1A) and DTY167 cells (*ycf1*Δ mutant, Figure 1B) to growth inhibition by 1-chloro-2,4-dinitrobenzene (CDNB). Cells were grown at 30°C for 24 hours to an OD_{600 nm} of approximately 1.4 in YPD medium before inoculation
20 of aliquots into 15 ml volumes of the same medium containing 0-60 μM CDNB. OD_{600 nm} was measured at the times indicated.

Figure 2 is a graph depicting the time course of [³H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in the absence (-MgATP) or presence of 3 mM MgATP (+MgATP) in
25 reaction media containing 66.2 μM [³H]DNP-GS, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol, and 25 mM Tris-MES (pH 8.0) at 25°C. Values shown are means ± S.E. (n = 3).

Figure 3 is a series of graphs depicting the kinetics of uncoupler-insensitive [^3H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Figure 3A: MgATP concentration-dependence of uncoupler-insensitive uptake. Figure 3B: DNP-GS concentration-dependence of MgATP-dependent uncoupler-insensitive uptake. The MgATP concentration-dependence of uptake was measured with 66.2 μM [^3H] DNP-GS. The DNP-GS concentration-dependence of uptake was measured with 3 mM MgATP. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5 μM gramicidin D. The kinetic parameters for vacuolar membrane vesicles purified from DTY165 cells were $K_{\text{m(MgATP)}}$ $86.5 \pm 29.5 \mu\text{M}$, $K_{\text{m(DNP-GS)}}$ $14.1 \pm 7.4 \mu\text{M}$, $V_{\text{max(MgATP)}}$ $38.4 \pm 5.6 \text{ nmol/mg/10 minutes}$, $V_{\text{max(DNP-GS)}}$ $51.0 \pm 6.3 \text{ nmol/mg/10 minutes}$. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *J. Soc. Ind. Appl. Math.* 11:431-441). Values shown are means \pm S.E. ($n = 3$).

Figure 4 is a series of graphs depicting sucrose density gradient fractionation of vacuolar membrane-enriched vesicles prepared from DTY165 cells. One ml (1.1 mg protein) of partially purified vacuolar membrane vesicles derived from vacuoles prepared by the Ficoll flotation technique were applied to a linear sucrose density gradient (10-40%, w/v) and analyzed for protein (Figure 4A), α -mannosidase activity (Figure 4B), V-ATPase activity (Figure 4C), and MgATP-dependent, uncoupler-insensitive [^3H]DNP-GS uptake (Figure 4D). [^3H]DNP-GS uptake and enzyme activity were assayed as described herein in Table 4 and the accompanying text.

Figure 5 includes a graph (Figure 5A) depicting the effect of transformation with pYCF1-HA or pRS424 on MgATP-dependent, uncoupler-insensitive [^3H]DNP-GS uptake by vacuolar membranes purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 66.2 μM [^3H]DNP-GS and 5 μM gramicidin D. Also shown (Figure 5B) is an image of a gel depicting immunoreaction of vacuolar membrane proteins prepared from pYCF1-HA-

transformed and pRS424-transformed DTY165 and DTY167 cells with mouse monoclonal antibody raised against the 12CA5 epitope of human influenza hemagglutinin. All lanes were loaded with 25 µg of delipidated membrane protein and subjected to SDS-polyacrylamide gel electrophoresis and Western analysis as described
5 herein. The M_r of YCF1-HA (boldface type) and the positions of the M_r standards are indicated on the figure.

Figure 6 is a series of graphs depicting transformation with pYCF1-HA (Figure 6A) or pRS424 (Figure 6B) on the sensitivity of DTY167 cells to growth retardation by CDNB. Cells were grown at 30°C for 24 hours to an $OD_{600\text{ nm}}$ of
10 approximately 1.4 in AHC medium (Kim *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:6128-6132) before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60 µM CDNB. $OD_{600\text{ nm}}$ was measured at the times indicated.

Figure 7 is a series of photomicrographs of DTY165 (Figures 7A and 7C) and DTY167 cells (Figures 7B and 7D) after incubation with monochlorobimane.
15 Cells were grown in YPD medium for 24 hours at 30°C and 100 µl aliquots of cell suspensions were transferred into 15 ml volumes of fresh YPD medium containing 100 µM monochlorobimane. After incubation for 6 hours, the cells were washed and examined in fluorescence (Figures 7C and 7D) or Nomarski mode (Figures 7A and 7B) as described herein.

Figure 8 is a series of graphs depicting uptake of Cd^{2+} into vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake of $^{109}Cd^{2+}$ by DTY165 membranes (Figure 8A) or DTY167 membranes (Figure 8B) was measured
20 in the absence of MgATP plus (O) or minus GSH (1 mM) (□) or in the presence of MgATP (3 mM) plus (●) or minus (■) GSH. $^{109}Cd_2SO_4$ and gramicidin-D were added at concentrations of 80 µM and 5 µM, respectively. Figure 8C: Rate of $^{109}Cd^{2+}$
25 uptake by DTY165 membranes plotted as a function of the total concentration of Cd^{2+} ($[Cd^{2+}]_{total}$) added to uptake medium containing 1 mM GSH, 3 mM MgATP and 5 µM gramicidin-D. Values shown are means \pm SE ($n = 3-6$).

Figure 9 is a series of graphs depicting purification of cadmium glutathione complexes by gel-filtration (Figure 9A) and anion-exchange chromatography (Figures 9B and 9C). Twenty mM $^{109}\text{Cd}_2\text{SO}_4$ was incubated with 40 mM GSH at 45°C for 24 hours and the mixture was chromatographed on Sephadex G-15 to resolve a high molecular weight ^{109}Cd -labeled component (HMW - $^{109}\text{Cd.GS}$) from a low molecular weight component (LMW - $^{109}\text{Cd.GS}$) (Figure 9A). The peaks corresponding to HMW - $^{109}\text{Cd.GS}$ and LMW - $^{109}\text{Cd.GS}$ were then chromatographed on Mono-Q and eluted with a linear NaCl gradient (---) (Figure 9B and 9C). ^{109}Cd (cpm $\times 10^{-3}$) was determined on 5 μl aliquots of the column fractions by liquid scintillation counting.

Figure 10 is a series of graphs depicting the kinetics of MgATP-dependent, uncoupler-insensitive $^{109}\text{Cd.GS}_2$ (HMW - $^{109}\text{Cd.GS}$, Figure 10A) and $^{109}\text{Cd.GS}$ (LMW - $^{109}\text{Cd.GS}$, Figure 10B) uptake. DNP-GS was added at the concentrations (μM) indicated to DTY165 membranes ($\bullet, \circ, \blacksquare, \square, \Delta$) or DTY167 membranes (\diamond). A secondary plot of the apparent Michaelis constants for Cd.GS_2 uptake ($K_m^{\text{app}}/\text{Cd.GS}_2$) as a function of DNP-GS concentration is shown (Figure 10C). The kinetic parameters for Cd.GS_2 transport by DTY165 membranes were K_m , $39.1 \pm 14.1 \mu\text{M}$, V_{max} , $157.2 \pm 30.4 \text{ nmol/mg/10 minutes}$ and $K_{i(\text{DNP-GS})}$, $11.3 \pm 2.1 \mu\text{M}$. Kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *supra*). Values shown are means \pm SE ($n = 6$).

Figure 11 is a graph depicting matrix-assisted laser desorption mass spectrometry (MALD-MS) of HMW - Cd.GS . MALD-MS was performed on Sephadex G-15-, Mono-Q-purified HMW - Cd.GS as described herein. The molecular structure inferred from a mean m/z ratio of 725.4 ± 0.7 ($n = 9$) and average Cd.GS stoichiometry of 0.5 [*bis*(glutathionato)cadmium, Cd.GS_2 , molecular weight 724.6 Da] is shown.

Figure 12 is an image of a gel depicting induction of *YCF1* expression and *YCF1*-dependent Cd.GS_2 and DNP-GS transport by pretreatment of DTY165 cells with CdSO_4 (Cd^{2+} , 200 μM) or CDNB (150 μM) for 24 hours. *YCF1*-specific mRNA and 18S rRNA were detected in the total RNA extracted from control or pretreated

cells (10 µg/lane) by RNase protection. Uptake of $^{109}\text{Cd.GS}_2$ (50 µM) or [^3H]DNP-GS (66.2 µM) by vacuolar membrane vesicles was measured in standard uptake medium containing 5 µM gramicidin-D. Values shown are means \pm SE ($n = 3$).

5 Figure 13A and 13B is the sequence of *AtMRP2* cDNA (SEQ ID NO:1). Lower case letters correspond to 5'- and 3'-untranslated regions (UTRs).

 Figure 14A-D is the genomic sequence of *AtMRP2* (SEQ ID NO:2). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

10 Figure 15 is the deduced amino acid sequence of *AtMRP2* (SEQ ID NO:3).

 Figure 16A and 16B is the sequence of *AtMRP1* cDNA (SEQ ID NO:4). Lower case letters correspond to 5'- and 3'-UTRs.

15 Figure 17A-D is the genomic sequence of *AtMRP1* (SEQ ID NO:5). Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-UTRs, respectively; lower case letters nested within the sequence correspond to introns.

 Figure 18 is the deduced amino acid sequence of *AtMRP1* (SEQ ID NO:6).

20 Figure 19 is a series of graphs depicting the time course and concentration-dependence of DNP-GS uptake in *AtMRP1*-transformed yeast. Figure 19A is a graph depicting the time course of [^3H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed or pYES3-transformed DTY168 cells. MgATP-dependent uptake was measured in reaction media containing 61.3 µM
25 [^3H]DNP-GS, 5 µM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown are means \pm SE ($n = 3$). Figure 19B is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive uptake of [^3H]DNP-GS by membrane vesicles purified from pYES3-*AtMRP1*.

transformed DTY168 cells. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5 μ M gramicidin-D. The kinetic parameters for uptake were $K_{m(\text{DNP-GS})}$ $49.7 \pm 15.4 \mu\text{M}$, V_{\max} $6.0 \pm 1.7 \text{ nmol/mg/10 minutes}$. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, supra). Values shown are means \pm SE (n = 3).

Figure 20 is a series of graphs depicting sensitivity of MgATP-dependent, uncoupler-insensitive [^3H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed DTY168 cells. Uptake was measured for 10 minutes in standard uptake medium containing the indicated concentrations of vanadate. In Figure 20A, there is a graph depicting total MgATP-dependent, uncoupler-insensitive [^3H]DNP-GS uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES-transformed DTY168 cells. In Figure 20B, there is a graph depicting *AtMRP1*-dependent uptake. I_{50} (exclusive of uninhibitable *AtMRP1*-independent component) = $8.3 \pm 3.2 \mu\text{M}$. Values shown are means \pm SE (n = 3).

Figure 21 is a series of graphs depicting the hydropathy alignment of *AtMRP2*, *AtMRP1*, *S. cerevisiae* YCF1 (*ScYCF1*), human *MRP1* (*HmMRP1*) and rat *cMOAT* (*RtCMOAT*).

Figure 22 is a diagram depicting domain comparisons between *AtMRP1*, *ScYCF1*, *HmMRP1*, *RtCMOAT*, rabbit *EBCR* (*RbEBCR*) and *HmCFTR*. The domains indicated are the N-terminal extension (NH_2), first and second sets of transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH).

Figure 23 is the promoter sequence of the *Arabidopsis AtMRP1* gene (SEQ ID NO:7). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

Figure 24 is the promoter sequence of the *Arabidopsis AtMRP2* gene (SEQ ID NO:8). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

5 Figure 25 is a graph depicting MgATP-dependence of [3 H]medicarpin uptake by vacuolar membrane vesicles before (Medicarpin/GSH) and after maize GST-mediated conjugation with GSH (Medicarpin-GS). [3 H]medicarpin or [3 H]medicarpin-GS was added at a concentration of 65 μ M. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Values shown are means \pm SE (n = 3).

10 Figure 26 is a graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive [3 H]medicarpin-GS uptake into vacuolar membrane vesicles. Uptake was allowed to proceed for 20 minutes in standard uptake medium containing 3 mM MgATP and 5 μ M gramicidin D. The kinetic parameters were K_m 21.5 \pm 15.5 μ M and V_{max} 77.8 \pm 23.3 nmol/mg/20 minutes. Values shown are means \pm SE (n = 3).

15 Figure 27 is a series of graphs depicting concentration-dependence of MgATP-dependent, uncoupler-insensitive C₃G-GS, IAA-GS and ABA-GS uptake by vacuolar membrane vesicles purified from *V. radiata* (Figure 27A) and *Z. mays* (Figure 27B). Uptake was allowed to proceed for 10 minutes in reaction medium containing 50 μ M GS-conjugate, 400 mM sorbitol, 3 mM MgATP, 50 mM KCl, 0.1% (w/v) BSA, 5 μ M gramicidin-D and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown
20 are means \pm SE (n = 3).

25 Figure 28 is an image of a photograph depicting the growth of wild type (WT) and *YCF1* transgenic *Arabidopsis* (*YCF1*) seeds on media containing CdSO₄ (200 μ M) or 1-chloro-2,4-dinitrobenzene (CDNB, 25 μ M). Transgenic plants were generated as described herein.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based upon the molecular identification of a new class of membrane transporter in yeast and plants, the GS-X pump. As a result of the present

invention, new insights into the membrane transport phenomena associated with heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation are evident. These insights provide a means, as is evident from the description of the present invention, for the manipulation of plants and the cells thereof, to affect heavy metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant pigmentation and bioremediation.

The process of "storage excretion" is a necessity for plants. Whereas mammals have the option of excreting GS-conjugates to the extracellular medium for elimination by the kidneys, plants are nearly totally reliant on the sequestration of noxious compounds in the central vacuole, which frequently accounts for 40-90% of total intracellular volume. Due to the virtual absence of specialized excretory organs and the presence of massive vacuoles in plants, a process (intracellular compartmentation) that is probably only an intermediate step in the elimination of xenobiotics from the cytosol of mammalian cells, is believed to constitute a terminal phase of detoxification in plants.

The data which are described herein establish that the yeast gene *YCF1* and two plant homologs of *YCF1*, *AtMRP1* and *AtMRP2*, isolated from *Arabidopsis thaliana*, each encode a vacuolar GS-X pump. The data further establish that the GS-X pump participates in herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions (exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins).

The plant *AtMRP1* and *AtMRP2* gene products use MgATP as an energy source for the transport of glutathionated derivatives of both endogenous and exogenous compounds in plants and thus, the discovery of these genes in the present invention is important at three levels. The identification of these genes and their encoded products represents the first identification of ABC transporters in plants for

which a biochemical function is defined. The discovery establishes, contrary to the prevailing chemiosmotic model for solute transport in plants, that many energy-dependent solute transport processes in plants are not driven by a transmembrane H⁺ electrochemical potential difference. Further, the identification and isolation of these genes and their encoded products permits a plant element, critical for removal of compounds from the cytosol that can form glutathionine S-conjugates, to be manipulated.

It has been discovered in the present invention that two plant genes, *AtMRP1* and *AtMRP2*, are the structural and functional homologs of the gene encoding yeast YCF1. Proteins encoded by plant *AtMRP1* and *AtMRP2* thus represent a new subclass of ATP binding cassette transporters.

It has been further discovered in the present invention that the yeast YCF1 protein, a GS-X pump, is capable of MgATP-energized transport of organic GS-conjugates and of MgATP-energized transport of cadmium upon complexation with GSH. In addition, when plants have introduced into the cells thereof the *YCF1* gene (a transgenic plant comprising *YCF1*), expression of *YCF1* therein confers upon the plants resistance to both inorganic and organic xenobiotics exemplified by cadmium and 1-chloro-2,4-dinitrobenzene, respectively.

Also discovered in the present invention is the fact that *AtMRP1* and *AtMRP2*, when expressed in a strain of yeast which is deficient in YCF1, can substitute for *YCF1* as a GS-X pump. In addition, transformation of plants by *YCF1* confers upon the plant properties which are characteristic of *YCF1* gene expression. Thus, it appears that *YCF1* and the *AtMRP* genes are essentially functionally interchangeable.

In addition, there is provided as part of the invention the promoter/regulatory sequences which control expression of the plant *AtMRP1* and *AtMRP2* genes of the invention. These promoter sequences are useful for the identification of compounds which affect expression of these genes in plants and for conferring on other genes the ability to respond to factors that modulate *AtMRP1* and/or *AtMRP2* expression.

Further discovered in the present invention is the fact that the plant GS-
X pump serves to facilitate the vacuolar storage of antimicrobial compounds induced
following the hypersensitive response to fungal pathogens in the healthy cells
surrounding fungally-induced lesions. Such a process is believed to limit the spread of
5 tissue damage by limiting propagation of the pathogen and spatially delimiting the
toxic action of the phytoalexin itself.

Ascription of specific enzymic and regulatory roles to most of the genes
of the anthocyanin biosynthetic pathway has been achieved by genetic and biochemical
studies of maize with one notable exception, the *Bronze-2* gene. It is known that the
10 characteristic coloration of *Bronze-2* (*bz2*) mutants is a consequence of the
accumulation of cyanidin-3-glucoside in the cytosol. However, in wild type (*Bz2*)
plants, anthocyanins are transported into the vacuole and become purple or red. In the
mutant (*bz2*) plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a
brown ("bronze") pigment. The biochemical basis for the accumulation of
15 anthocyanins in the cytosol is not known. However, Marrs *et al.*, (1995, *supra*) have
discovered that *Bz2* encodes a glutathione S-transferase which is responsible for
conjugating anthocyanin with GSH. It has now been discovered in the present
invention that the plant GS-X pump is the entity responsible for the delivery of
glutathionated anthocyanins into the vacuole.

20 Identification of the GS-X pump at the molecular level has served to
confirm its wide distribution and demonstrate that these transporters constitute a
multigene family within the ABC transporter superfamily. The critical finding was that
overexpression of the human multidrug resistance-associated protein (*MRP1*) gene
(Cole *et al.*, 1992, *supra*) confers increased MgATP-dependent GS-conjugate transport
25 (Muller *et al.*, 1994 *supra*; Leier *et al.*, 1994, J. Biol. Chem. 269:27807-27810).

Several other closely related GS-X pump genes have been characterized.
For example, a liver-specific GS-X pump (*cMOAT*), mutation of which is believed to
cause hereditary hyperbilirubinemia, has been cloned (Paulusma *et al.*, 1996, Science
271:1126-1128). The present invention establishes that YCF1 is a GS-X pump. In

addition, as will become apparent from a reading of the present description, two plant genes, *AtMRP1* and *AtMRP2* have been discovered in the present invention to encode homologs of MRP1, YCF1 and cMOAT.

5 The identification of YCF1 as a vacuolar GS-X pump is described in detail in the experimental details section. Similarly, the identification of two plant homologs of YCF1, *AtMRP1* and *AtMRP2*, is also described in detail in the experimental details section. Once armed with the present invention, the skilled artisan will know how to identify and isolate genes encoding other plant GS-X pumps involved in sequestration of a variety of compounds in plants by following the procedures
10 described herein.

A plant gene encoding a GS-X pump is isolated using any one of several known molecular procedures. For example, primers comprising conserved regions of the sequences of any of *YCF1*, *AtMRP1* or *AtMRP2*, or in fact primers comprising conserved regions of any MRP subclass (*i.e.*, probes directed to human *MRP1 cMOAT*,
15 and other MRP genes) may be used as probes to isolate, by polymerase chain reaction (PCR) or by direct hybridization, as yet unknown *YCF1*, *AtMRP1* or *AtMRP2* homologs in a DNA library comprising specific plant DNAs. Alternatively, antibodies directed against YCF1, *AtMRP1* or *AtMRP2* may be used to isolate clones encoding a GS-X pump from an expression library comprising specific plant DNAs. The isolation
20 of primers, probes, molecular cloning and the generation of antibodies are procedures that are well known in the art and are described, for example, in Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York) and in Harlow *et al.* (1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor, New York).

25 The invention includes an isolated DNA encoding a plant GS-X pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell. Preferably, the DNA encoding a plant GS-X pump is at least about 40% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-X pump is at least

about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-X pump is *Arabidopsis AtMRP1* or *AtMRP2*. Most preferably, the isolated DNA encoding a plant GS-X pump is SEQ ID NOS:1, 2, 4 or 5.

Thus, the invention should be construed to include genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes.

By "GS-X pump" as used herein, is meant a protein which transports a glutathione-conjugated compound across a biological membrane.

By the term "DNA encoding a GS-X pump" as used herein is meant a gene encoding a polypeptide capable of transporting a glutathionated compound across a biological membrane.

By "*AtMRP*-related gene" as used herein, is meant a gene encoding a GS-X pump which is a member of the *MRP/YCF1/cMOAT* family of genes. An *AtMRP1* or *AtMRP2*-related gene may be present in a cell which also encodes an *AtMRP* gene or it may be present in a different cell and in a different plant species.

As described in the Experimental Detail section, *AtMRP* genes encode proteins which have specific domains located therein, namely, the N-terminal extension, transmembrane spans, TM1 and TM2, nucleotide binding folds, NBF1 and NBF2, putative CFTR-like regulatory domain (R) and the C-terminus. An *AtMRP*-related gene is therefore also one in which selected domains in the related protein share significant homology (at least about 40% homology) with the same domains in either of *YCF1*, *AtMRP1* or *AtMRP2*. For example, when the R-domain in the *AtMRP*-related protein shares at least about 40% homology with the R domain in *YCF1*, *AtMRP1* or *AtMRP2*, and when the product of that is a GS-X pump, then that gene is an *AtMRP*-related gene. Similarly, when the N-terminal extension in the *AtMRP*-related protein shares at least about 40% homology with the N-terminal extension in

YCF1, AtMRP1 or AtMRP2, and when the product of that is a GS-X pump, then that gene is an *AtMRP*-related gene. It will be appreciated that the definition of an *AtMRP*-related gene encompasses those genes having at least about 40% homology in any of the described domains contained therein with the same or a similar domain in either of YCF1, AtMRP1 or AtMRP2. In addition, when the term homology is used herein to refer to the domains of these proteins, it should be construed to be applied to homology at both the nucleic acid and the amino acid levels.

While a significant homology between similar domains in *AtMRP*-related genes or their protein products is considered to be at least about 40%, preferably, the homology between domains is at least about 50%, more preferably, at least about 60%, even more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% and most preferably, the homology between similar domains is about 99% between a domain in an *AtMRP*-related gene or protein product thereof, and at least one of YCF1, *AtMRP1* or *AtMRP2* or the protein products thereof.

Plants from which *AtMRP1*, *AtMRP2* or YCF1 related genes may be isolated include any plant in which the GS-X pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

By the term "glutathionated-conjugated compound" as used herein is meant a compound, *e.g.*, a metal, a xenobiotic, a isoflavonoid phytoalexin, anthocyanin or auxin, which is chemically conjugated to glutathione. Conjugation of compounds to glutathione occurs naturally within cells and organisms and may also be accomplished enzymatically or non-enzymatically *in vitro* as described herein in the experimental details section.

Also included in the invention is an isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump. Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is at least about 40% homologous to a biologically active polypeptide fragment

of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90%, and even more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is about 200 nucleotides in length. More preferably, the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X pump is about 400 nucleotides, even more preferably, at least about 600, yet more preferably, at least about 800, even more preferably, at least about 1000, and more preferably, at least about 1200 nucleotides in length.

The invention further includes a vector comprising a gene encoding a plant GS-X pump and a vector comprising nucleic acid sequence encoding a biologically active fragment thereof. The procedures for the generation of a vector encoding a plant GS-X pump, or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (e.g., pBIN19) containing the target gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Lagrimini *et al.*, 1990, Plant Cell 2:7-18) or its endogenous promoter (Bevan, 1984, Nucl. Acids Res. 12:8711-8721).

Also included in the invention is a cell comprising an isolated DNA encoding a plant GS-X pump and a cell comprising an isolated DNA encoding a biologically active fragment thereof. Such a cell is referred to herein as a "recombinant cell."

The procedures for the generation of a cell encoding a plant GS-X pump or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook *et al.* (*supra*). Suitable cells include, but are not limited to, yeast cells, bacterial cells, mammalian cells, and baculovirus-
5 infected insect cells transformed with the gene for the express purpose of generating GS-X polypeptide. In addition, plant cells transformed with the gene for the purpose of producing cells and regenerated plants having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration.

10 The invention also includes an isolated preparation of a polypeptide comprising a plant GS-X pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is at least about 30% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a
15 polypeptide comprising a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least about 60%, even more preferably, at least about 70%, more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation
20 of a polypeptide comprising a plant GS-X pump is *Arabidopsis* AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is SEQ ID NOS: 3 or 6.

Also included in the invention is an isolated preparation of a
25 biologically active polypeptide fragment of a plant GS-X pump. Preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 30% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least

about 60%, even more preferably, at least about 70% and yet more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a
5 biologically active polypeptide fragment of a plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the polypeptide in the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is about 60 amino acids in length. More preferably, the polypeptide in the isolated preparation of a biologically active
10 polypeptide fragment of a plant GS-X pump is about 130 amino acids, even more preferably, at least about 200, yet more preferably, at least about 300, even more preferably, at least about 350, and more preferably, at least about 400 amino acids in length.

As used herein, the term "homologous" refers to the subunit sequence similarity between two polymeric molecules e.g., between two nucleic acid molecules, e.g., between two DNA molecules, or two polypeptide molecules. When a subunit
15 position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two polypeptide molecules is occupied by phenylalanine, then they are homologous at that position. The homology between two sequences is a direct
20 function of the number of matching or homologous positions, e.g., if half (e.g., 5 positions in a polymer 10 subunits in length) of the positions in two polypeptide sequences are homologous then the two sequences are 50% homologous; if 70% of the positions, e.g., 7 out of 10, are matched or homologous, the two sequences share 70%
25 homology. By way of example, the polypeptide sequences ACDEFG and ACDHIK (SEQ ID NOS:9 and 10, respectively) share 50% homology and the nucleotide sequences CAATCG and CAAGAC share 50% homology.

An "isolated DNA," as used herein, refers to a DNA sequence which has been separated from the sequences which flank it in a naturally occurring state, e.g., a DNA fragment which has been removed from the sequences which are normally

adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

As used herein, the term "isolated preparation of a polypeptide" describes a polypeptide which has been separated from components which naturally accompany it. Typically, a polypeptide is isolated when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, even more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) of a sample is the polypeptide of interest. The degree of isolation of the polypeptide can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis. For example, a polypeptide is isolated when it is essentially free of naturally associated components or when it is separated from the native compounds which accompany it in its natural state.

As used herein, by the term "biologically active" as it refers to GS-X pump activity as used herein, is meant a polypeptide, or a fragment thereof, which is capable of transporting a glutathionated compound across a biological membrane.

In summary, the invention should be construed to include DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof, which encode GS-X pump biological activity.

The invention further features an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS-X pump gene, wherein the nucleic acid is capable of inhibiting expression of the GS-X pump gene when introduced into cells comprising the GS-X pump gene. The nucleic acid is antisense to
5 either a portion or all of a plant GS-X pump gene, which gene is preferably *Arabidopsis AtMRP1*, *Arabidopsis AtMRP2* or a homolog thereof. The "isolated preparation of a nucleic acid" and the "portion" of the gene to which the nucleic acid is antisense, should be of a sufficient length so as to inhibit expression of the desired target gene. The actual length of the isolated preparation of the nucleic acid may vary, and will
10 depend on the particular target gene and the region of that gene which is targeted. Typically, the isolated preparation of the nucleic acid will be at least about 15 contiguous nucleotides; more typically, it will be between about 15 and about 50 contiguous nucleotides, or it may even be more than 50 contiguous nucleotides in length.

15 As used herein, a sequence of a nucleic acid is "antisense" to a portion or all of a GS-X pump gene when the sequence of nucleic acid does not encode a GS-X polypeptide. Rather, the sequence which is being expressed in the cells is identical to the non-coding strand of the GS-X pump gene and thus, does not encode a GS-X pump polypeptide.

20 "Complementary," as used herein, refers to the subunit sequence complementarity between two nucleic acids, e.g., two DNA molecules. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are
25 complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs).

In yet another aspect of the invention, there is provided an antibody directed against a plant GS-X pump, preferably AtMRP1 or AtMRP2, which antibody

is specific for the whole molecule or either the N-terminal or the C-terminal or internal portions of AtMRP1 or AtMRP2. Methods of generating such antibodies are well known in the art and are described, for example, in Harlow *et al.* (*supra*).

5 The present invention also provides for analogs of proteins or peptides encoded by *AtMRP1* or *AtMRP2*. Analogs can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter
10 its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;
valine, isoleucine, leucine;
aspartic acid, glutamic acid;
15 asparagine, glutamine;
serine, threonine;
lysine, arginine;
phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in*
20 *vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also
25 embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a

therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

5 The invention further includes a transgenic plant comprising an isolated DNA encoding a plant GS-X pump polypeptide or a fragment thereof, capable of transporting a glutathionated compound across a biological membrane. The transgenic plant of the invention may comprise a transgene encoding a plant GS-X pump polypeptide or a fragment thereof, or it may comprise a transgene encoding a yeast GS-X pump polypeptide or a fragment thereof, which yeast transgene is expressed in the
10 plant to yield a biologically active GS-X pump protein product. By way of example, there is provided herein in the experimental examples section a transgenic *Arabidopsis* plant comprising a yeast *YCF1* transgene, which when the transgene is expressed in the transgenic plant, confers upon the plant the ability to grow on media containing
15 concentrations of heavy metal (cadmium) or organic xenobiotic (CDNB) that otherwise prevent of nontransgenic plants.

 The invention also includes a transgenic plant comprising an isolated DNA comprising the sequence of a plant GS-X pump polypeptide or a fragment thereof, which plant GS-X pump is capable of transporting a glutathionated compound
20 across a membrane derived from a cell, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

 Thus, included in the invention is a transgenic plant comprising an isolated DNA encoding a yeast *YCF1* or a fragment thereof, capable of transporting a glutathionated compound across a membrane derived from a cell.
25

 In addition, the invention includes a transgenic plant comprising an isolated DNA comprising the sequence of a yeast *YCF1* gene or a fragment thereof, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

By "transgenic plant" as used herein, is meant a plant, the cells, the seeds and the progeny of which comprise a gene inserted therein, which gene has been manipulated to be inserted into the cells of the plant by recombinant DNA technology. The manipulated gene is designated as a "transgene."

5 By the term "nontransgenic but otherwise substantially homozygous wild type plant" as used herein, is meant a nontransgenic plant from which the transgenic plant was generated.

"Positioned in an antisense orientation with respect to the direction of transcription of the DNA" as used herein, means that the transcription product of the DNA, the resulting mRNA, does not encode a GS-X pump. Rather, the mRNA comprises a sequence which is complementary to an mRNA which encodes a GS-X pump.

15 If vacuolar transport rate limits xenobiotic detoxification and if the amount of GS-X pump is rate limiting on the overall rate of vacuolar uptake, transgenic plants with increased *YCF1*, *AtMRP1* or *AtMRP2* expression are expected to be more resistant to the toxic effects of glutathione-conjugable xenobiotics and capable of accumulating higher vacuolar conjugate levels than non-transgenic plants. The former property permits the sustained growth of transgenic plants in the presence of xenobiotic concentrations that would retard the growth of plants exhibiting normal levels of transporter expression. The latter property confers on the plants the ability for hyperaccumulation of glutathionated xenobiotics.

20 Increased resistance to xenobiotics has application in herbicide technology and plant growth in habitats polluted with organics. Hyperaccumulation has application in the extraction of organic pollutants from contaminated ground soils.

25 The closest known similar technologies to those described herein (a) involve the isolation of mutants or the engineering of plants in which the target for xenobiotic action is no longer sensitive, (b) involve the generation of mutants with elevated cellular levels of glutathione (GSH) or with increased glutathione-S-transferase activities, or (c) involve the application of chemical agents ("safeners") that

elevate GSH and/or glutathione-S-transferase levels or activities. These known technologies differ from the strategy proposed herein in three respects: (i) The utility of mutated target gene products is limited in its application to those xenobiotics that directly interact with the target in question. In contrast, the vacuolar GS-X pump is of broad substrate specificity. (ii) Technologies based on elevated cellular GSH levels or increased glutathione-S-transferase catalytic efficiencies are limited by the capacity of cells to subsequently metabolize and/or sequester the conjugates generated. The success of these latter technologies eventually depends on delivery of GSH-conjugates into the vacuole and in turn, depends on the activity of the vacuolar GS-X pump. (iii) Since the plant vacuole frequently constitutes 40-90% of total intracellular volume and the GS-X pump mediates the uptake of xenobiotics into this compartment, the potential for hyperaccumulation on a tissue weight basis is great. Hyperaccumulators may therefore be used for the fixation/sequestration of toxins and their removal from soils. None of the other known technologies have this characteristic.

The generation of transgenic plants comprising sense or antisense DNA having the sequence of a GS-X pump or a fragment thereof, may be accomplished by transformation of the plant with a plasmid encoding the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids containing a sense or antisense strand placed under the control of the strong constitutive CaMV 35S promoter or under the control of an inducible promoter (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene* 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired gene is known and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the Agrobacterium-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual* A5:1).

5 A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein. Northern blot hybridization analysis or PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the
10 cells of the transgenic plant express GS-X polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-X pump or fragments thereof. The procedures for performing such analyses are well known in the art and are described, for example, in Sambrook *et al.* (*supra*).

15 The transgenic plants of the invention are useful for the manipulation of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For example, a transgenic plant encoding an *AtMRP1* or an *AtMRP2* gene or an *AtMRP1*- or *AtMRP2*-related gene, or a yeast *YCF1* or *YCF1*-related gene, is useful for
20 xenobiotic detoxification and heavy metal detoxification when grown on soil containing xenobiotics or heavy metals. Such plants are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

25 Accordingly, the invention includes a method of removing xenobiotic toxins from soil comprising generating a transgenic plant having a transgene encoding a GS-X pump and planting the plant or the seeds of the plant in the soil wherein xenobiotic toxins in the soil are sequestered within the plant during growth of the plant in the soil.

Similarly, the invention includes a method of removing heavy metals from soil comprising generating a transgenic plant having a transgene encoding a GS-X pump and planting the plant or the seeds of the plant in the soil wherein heavy metals in the soil are sequestered within the plant during growth of the plant in the soil.

5 When the levels of xenobiotic toxins or heavy metals in the soil have been sufficiently reduced, the transgenic plant may be removed from the soil and destroyed or discarded in an environmentally safe manner. For example, the harvested plants can be reduced in volume and/or weight by thermal, microbial, physical or chemical means to decrease handling, processing and potential subsequent land filling costs (Cunningham *et al.*, 1996, *Plant Physiol.* 110:715-719). In the case of valuable
10 metals, subsequent smelting and recovery of the metal may be cost-effective (Raskin, 1996, *Proc. Natl. Acad. Sci. USA* 93:3164-3166).

This technique of remediating soil is more efficient, less expensive and easier than most chemical or physical methods. The estimated costs of remediation are
15 as follows: U.S. \$10-100 per cubic meter of soil for removal of volatile or water soluble pollutants by *in situ* remediation using plants; U.S. \$60-300 per cubic meter of soil for landfill or low temperature thermal treatment remediation of soil contaminated with the same compounds; and, U.S. \$200-700 per cubic meter of soil for remediation of soil contaminated with materials requiring special landfilling arrangements or high
20 temperature thermal treatment (Cunningham *et al.*, 1995, *Trends Biotechnol.* 13:393-397).

Preferably, the transgene in the transgenic plant of the invention is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of *AtMRP1*, *AtMRP2* or *YCF1*, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2*,
25 *YCF1*.

The types of plants which are suitable for use in this method of the invention include, but are not limited to, high yield crop species for which cultivation practices have already been perfected, or engineered endemic species that thrive in the area to be remediated.

In certain situations, it may be necessary to prevent the removal of substances such as xenobiotic toxins and heavy metals from the soil. In such situations, transgenic plants are generated comprising a transgene comprising a GS-X pump sequence which is in the antisense orientation with respect to transcription. Such transgenes therefore serve to inhibit the function of a GS-X pump expressed in the plants thereby preventing removal of xenobiotics or heavy metals from the soil.

The production of plants having GS-X pump antisense sequences has application in the manipulation of plant/food coloration and in the diminution of organic xenobiotic (e.g., herbicide) or heavy metal accumulation by crop species. For example, ingestion by animals or humans of low organic toxin/low heavy metal crops will likely contribute to an improvement in the overall health of animals and humans.

Accordingly, the invention includes a method of preventing the removal of xenobiotic toxins or heavy metals from soil comprising generating a transgenic plant having a transgene comprising a GS-X pump sequence which is in the antisense orientation with respect to transcription and planting the plant or the seeds of the plant in the soil, wherein removal of xenobiotics and heavy metals from the soil is prevented during growth of the plant in the soil.

The antisense sequences which are useful for the generation of transgenic plants having antisense GS-X pump sequences are those which will inhibit expression of a resident GS-X gene in the plant.

The types of plants which are suitable for use in this method of the invention using antisense sequences include, but are not limited to, plants for which anthocyanins contribute to flower, fruit or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable.

In a similar manner to that described herein, a transgenic plant may be generated which exhibits increased accumulation and/or resistance to isoflavonoid alexins by introducing into the cells of the plant a transgene encoding a GS-X pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant, thereby isolating the isoflavonoid alexins from the cytoplasm of the cells of the plant.

Preferably, the transgene is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or analogs of *AtMRP1*, *AtMRP2* or *YCF1*, or the transgene is a gene which is related to *AtMRP1*, *AtMRP2* or *YCF1*.

5 The invention thus includes a method of generating a pathogen-resistant transgenic plant comprising introducing into the plant a transgene encoding a GS-X pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant.

The types of plants suitable for the introduction of the desired transgene include, but are not limited to, plants which are leguminous plants, for example, alfalfa,
10 cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut.

As discussed herein, it has also been discovered in the present invention that the *Bz2* gene which encodes a glutathione-S-transferase, glutathionates anthocyanins and possibly other compounds for transport by the GS-X pump. The
15 anthocyanin-derivatives so generated are subsequently transported across biological membranes by the vacuolar GS-X pump. Vacuolar anthocyanins are responsible for the red and purple hues of many plant organs (petals, leaves, stems, seeds, fruits, etc.). Vacuolar anthocyanins are found in most flowering plants. However, they are not solely responsible for plant coloration. Rather, plant coloration is determined by the
20 relative amounts and combinations in which these various pigments are accumulated. Thus, it is possible to manipulate plant coloration by generating transgenic plants with increased (sense DNA) or decreased (antisense DNA) expression of the GS-X pump. Transgenic plants having GS-X pump sense sequences are expected to contain more red/purple pigmentation than their nontransgenic but otherwise homozygous
25 counterparts and transgenic plants having GS-X pump antisense sequences are expected to contain less red/purple pigmentation and possibly more brown pigmentation than their nontransgenic but otherwise homozygous counterparts. The generation of such types of transgenic plants may be accomplished following the procedures described herein.

With respect to the aforementioned information regarding anthocyanins, it is important to note that accumulating evidence from studies of the MRP-subclass members from non-plant sources reveals that the group of transporters formerly referred to as GS-X pumps because of their affinity toward GS-conjugates, GSSG and cysteinyl leukotrienes, do not transport GS-conjugates exclusively (Ishikawa et al., 1997, Bioscience Reports 17:189-208). Investigation of the human MRP1 protein, cMOAT and ScYCF1 establish that these proteins are capable of transporting a broad range of compounds in addition to GS-conjugates and GSSG Jedlitschky et al., 1996, Cancer Res. 56:988-994; Paulusma et al., 1996, Science 271:1126-1128; Jansen et al., 1987, Hepatol. 7:71-76; Sathirakul et al., 1993, J. Pharmacol. Exp. Therap. 268:65-73). Thus, these proteins transport non-glutathionated compounds.

It has been discovered in the present invention that the plant proteins, AtMRP1 and AtMRP2, differ in their substrate preferences. For example, not only does AtMRP2 exhibit a much higher transport capacity than does AtMRP1, but AtMRP2 has the capacity to transport chlorophyll breakdown products in leaf senescence, which breakdown products are not glutathionated. Thus, according to the present invention, it is possible to manipulate plant coloration by changing the relative levels of expression of various members of this class of transporters in a plant cell. It is possible, using the information provided herein, to affect the rate of breakdown of chlorophyll, for example, by manipulating the expression of AtMRP2 in a plant cell.

In addition to the above, there is provided as part of the invention, *AtMRP1* and *AtMRP2* promoter sequences. By operably coupling the *AtMRP1* or *AtMRP2* promoters to other genes, it may be possible to confer on these other genes expression characteristics similar to those of *AtMRP1* or *AtMRP2*, namely, modulation by xenobiotics, plant pathogens, etc. The data which are presented herein include the promoter sequences of these genes, which promoter sequences are useful in a variety of applications in plants. For example, GS-X pump activity which is associated with herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions

(exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins) may be examined as a result of the present invention. The present invention facilitates the identification of plants and
5 cells therein which are capable of GS-X pump activity, and further facilitates the exploitation of plant cell GS-X pump activity for the purpose of affecting plant function with respect to herbicide metabolism, heavy metal sequestration, plant-pathogen interactions, plant cell pigmentation and plant hormone metabolism.

The invention includes an isolated DNA comprising a plant GS-X pump
10 promoter sequence capable of driving expression of a plant GS-X pump gene, which gene is capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell.

Preferably, the isolated DNA comprising a plant GS-X pump promoter
15 sequence is at least about 40% homologous to at least one of the *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. More preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at
20 least one of *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. Most preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is *Arabidopsis AtMRP1* or *AtMRP2* as shown in Figures 1 and 2, respectively.

Thus, the invention should be construed to include isolated DNA
25 sequences comprising promoter sequences which in their natural form drive expression of genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes. Once armed with the present invention, it is a simple matter to isolate sequences which are related to those shown in Figures 1 and 2. For example, conventional hybridization technology and/or PCR technology may be

employed, primers may be designed using the sequences provided herein, data bases may be searched and the like. Procedures for the isolation of promoter sequences which are related to those described herein are described in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY) and in Ausubel
5 *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

By the term "promoter sequence" as used herein, is meant a DNA sequence which is required for expression of a gene which is operably linked thereto. In some instances, this sequence may be a core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory
10 elements which are required for expression of the gene in a tissue-specific manner. Thus, a promoter sequence must include an RNA polymerase binding site and may include appropriate transcription factor binding sites as are necessary for activation of transcription and expression of the gene to which the promoter sequence is attached at the 5' end of the gene.

15 Typically, the promoter sequence of the invention comprises at least about 150 bp in length. More typically, the promoter sequence comprises at least about 300 bp in length. More typically, the promoter sequence comprises at least about 400 bp, even more typically, at least about 500 bp, yet more typically, at least about 600 bp, even more typically, at least about 800 bp, yet more typically, at least about 1000 bp
20 and even more typically, at least about 1200 or more bp in length.

The promoter sequence of the invention may also comprise discrete sequences (elements) which function to regulate the activity of the promoter. Frequently, such elements respond to the presence or absence of environmental factors, thereby controlling gene expression in direct response to factors which are associated
25 with the environmental milieu of the plant. The response of the plant to these factors affects the overall well-being of the plant. Elements which may be present in the promoter sequence of the invention include, but are not limited to, a Myb recognition sequence, a xenobiotic regulatory element, an antioxidant response element, a bZIP recognition sequence, and the like.

Plants from which *AtMRP1*- or *AtMRP2*-related genes and therefore promoter sequences, may be isolated include any plant in which the GS-X pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

5 The invention further includes a vector comprising a plant GS-X pump promoter sequence operably fused to a reporter gene and capable of driving expression of the reporter gene. The procedures for the generation of a vector comprising a plant GS-X pump promoter sequence are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable vectors
10 include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (e.g., pBIN19) (Lagrimini et al., 1990, *Plant Cell* 2:7-18; Bevan, 1984, *Nucl. Acids Res.* 12:8711-8721).

 Also included in the invention is a cell comprising a plant GS-X pump promoter sequence operably fused to a reporter gene. The procedures for the generation
15 of a cell encoding a plant GS-X pump or fragment thereof, are well known in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable cells include, but are not limited to, plant cells, yeast cells, bacterial cells, mammalian cells, and baculovirus-infected insect cells. In addition, plant cells transformed with the promoter/reporter gene construct, for the purpose of
20 assessing the effect of various compounds on promoter activity are also contemplated in the invention. Normal plant cells and those plant cells having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration, which cells comprise the promoter sequence of the invention operably fused
25 to a reporter gene, are all contemplated as part of the invention. When the promoter is fused to a reporter gene, the promoter is said to be operably linked to the reporter gene.

 A "reporter gene" as used herein, is one which when expressed in a cell, results in the production of a detectable product in the cell. The level of expression the

product in the cell is proportional to the activity of the promoter sequence which drives expression of the reporter gene.

By describing two nucleic acid sequences as "operably linked" as used herein, is meant that a single-stranded or double-stranded nucleic acid moiety
5 comprises each of the two nucleic acid sequences and that the two sequences are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

Suitable reporter genes include, but are not limited to, β -glucuronidase
10 (GUS) and green fluorescent protein (GFP), although any reporter gene capable of expression and detection in plant cells which are either known or heretofore unknown, may be fused to the plant GS-X promoter sequences of the invention.

The invention further includes a transgenic plant comprising an isolated DNA comprising a plant GS-X pump promoter sequence as defined herein.

15 The generation of transgenic plants comprising a plant GS-X pump promoter sequence operably fused to a reporter gene, may be accomplished by transformation of the plant with a plasmid comprising the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene*
20 72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired nucleic acid is known and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those
25 of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the *Agrobacterium*-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual* A5:1).

A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein.

5 Northern blot hybridization analysis or RT-PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the cells of the transgenic plant express GS-X polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-X pump or fragments thereof. The procedures for

10 performing such analyses are well known in the art and are described, for example, in Sambrook et al. (*supra*).

The transgenic plants of the invention are useful for the examination of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For

15 example, a transgenic plant comprising an *AtMRP1* or an *AtMRP2* promoter sequence fused to a reporter gene is useful for the examination of xenobiotic detoxification and heavy metal detoxification when grown on soil having xenobiotic toxins or heavy metals. Such plants are useful to an understanding of the mechanisms by which GS-X pump gene expression is activated and are therefore useful for the eventual generation

20 of plants which are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

The types of plants which are suitable for use include, but are not limited to, high yield crop species for which cultivation practices have already been

25 perfected, or engineered endemic species that thrive in the area to be remediated. In addition plants for which anthocyanins contribute to flower or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable are also suitable for use in the invention. Further useful plants are those in which it is desirable that they are capable of increased accumulation and/or resistance

to isoflavonoid alexins. Plants for which pathogen resistance is desired are also useful in the invention. Such plants include, but are not limited to, plants which are leguminous plants, for example, alfalfa, cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut. In addition, plants for which it is desirable to manipulate plant coloration are also useful in the invention.

The promoter sequences of *Arabidopsis* GS-X pump genes *AtMRP1* and *AtMRP2* are shown in Figures 23 and 24, respectively. The following should be noted. bZIP transcription factor recognition elements have the sequences CACGTG or TGACG(T/C). One of these is present in the *AtMRP2* promoter sequence, but none are present in the *AtMRP1* promoter sequence. Myb transcription factor recognition elements having the sequences A(a/D)(a/D)C(G/C) and AGTTAGTTA, wherein a/D = A, G or T with A being preferred, are present in the *AtMRP1* promoter sequence, but are not present in the *AtMRP2* promoter sequence. Xenobiotic regulatory elements (XREs) having the core sequence GCGTG are found in multiple copies in the promoters of cytochrome P450 monooxygenase genes and glutathione S-transferase genes (Rushmore et al., 1993, J. Biol. Chem. 268:11475-11478). One XRE is found in the promoter sequence of *AtMRP1*. Antioxidant response elements (AREs) consist typically of two core sequences GTGACA(A/T)(A/T)GC (SEQ ID NO:11) that are binding sites for Activator Protein-1 (AP-1) transcription factor complex (Daniel, 1993, CRC Crit. Rev. Biochem 25:173-207; Friling et al., 1992, Proc. Natl. Acad. Sci. USA 89:668-672). There is only one ARE in the *AtMRP1* promoter sequence shown in Figure 23. It has been proposed that GST genes containing an ARE are induced by electrophiles and conditions that generate oxidative stress (Daniel, *supra*). RNA instability determinants having the sequence ATTTA have been found in several plant GSTs. These sequences, considered to target RNAs for degradation by RNases are usually found in the 3'-UTRs of genes (Takahashi et al., 1992, Proc. Natl. Acad. Sci. USA 89:56-59). Several of these sequences are found in both the *AtMRP1* and *AtMRP2* promoter sequences presented herein. However, it is not clear whether these sequences merely reflect the AT-richness of the sequences.

To assess GS-X pump gene expression in a plant cell whether the cell is contained within a plant or whether the cell is separated from the plant, a plasmid may be generated which comprises the β -glucuronidase (GUS) reporter gene fused to a plant GS-X promoter sequence. Preferably, the promoter sequence is either *AtMRP1* or *AtMRP2*. The appropriate restriction fragment is subcloned into the GUS
5 expression vector pBI101.3 (Jefferson et al., 1987, EMBO J., 6:3901-3907). After confirming the correct reading frame by sequencing, *Agrobacterium* or any other suitable vector, is transformed with the expression construct and is then used to transform the plant, or the cells thereof (Valvekens et al., 1988, Proc. Natl. Acad. Sci.
10 USA 85:5536-5540).

Expression of GUS may be localized histochemically by staining with 5-bromo-4-chloro-3-indoyl β -D-glucuronide (X-Gluc) (Jefferson et al., *supra*). Sections are obtained from the plant, they are incubated in X-Gluc, cleared by boiling in ethanol and are examined under the microscope. To eliminate or enumerate
15 complications arising from the transfer of GUS reaction product between cells, the distribution of GUS expression is then further examined both immunologically and biochemically. β -glucuronidase protein is assessed using standard dot-blotting and immunolocalization techniques (Harlow et al., 1988, *Antibodies: A Laboratory*
20 *Manual*, Cold Spring Harbor Laboratory, NY) using rabbit anti- β -glucuronidase serum (Clontech). Direct estimates of GUS activity are be made fluorimetrically using 4-methyl-umbelliferyl glucuronide as substrate (Jefferson et al., *supra*) after dissection and extraction of explants.

GUS reporter gene analyses enable examination of plant responses to oxidative stress and pathogens as well as herbicides. In addition, GUS reporter gene
25 analyses enable tests of whether certain pigment-rich cell types also exhibit high levels of *AtMRP* expression.

The *AtMRP1* and *AtMRP2* promoter sequences are also useful for manipulating the expression of other genes in plants in that, transgenic plants may be generated which contain a desired plant gene operably fused to a GS-X pump promoter

sequence. The GS-X pump promoter sequence may be an *AtMRP1* or an *AtMRP2* promoter sequence or a *YCF1* promoter sequence positioned in an orientation such that the promoter sequence drives expression of the desired gene. The desired gene may be a plant or a non-plant gene. The generation of such transgenic plants confers upon the plants the ability to respond to the presence of xenobiotics and other compounds which influence the promoter activity

In considering transport substrates for GS-X pumps, the status of GSSG as an endogenous GS-conjugate (of GSH with itself) and its involvement in cellular responses to active oxygen species (AOS) should not be overlooked. The sulfhydryl group of GSH confers strong nucleophilicity and the facility for reacting with AOS, such as superoxide radicals ($O_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}) and hydrogen peroxide. GSH is found in the majority of eukaryotes but in prokaryotes (eubacteria) it appears to be restricted to the cyanobacteria and purple bacteria (Fahey and Sundquist 1991, Adv. Enzymol. Relat. Mol. Biol. 64:1-53). Since the cyanobacteria are considered to be the first group of organisms capable of oxygenic photosynthesis and these and the purple bacteria probably gave rise to plant chloroplasts and mitochondria, respectively, it has been proposed that the emergence of the capacity for GSH biosynthesis was associated with the appearance of oxygenic and oxytrophic metabolism (approximately 4×10^9 years ago) to combat the attendant problem of AOS production. Most, if not all, of the factors known to elicit GST induction - pathogen attack, heavy metals, certain organic xenobiotics, wounding and ethylene - promote AOS production (Inze and Montagu 1995, Current Opinion in Biotech. 6:153-158). Intriguing, therefore, is the possibility that GS-X pumps arose from the need to detoxify AOS and the products of their action.

The feasibility of such a scheme has yet to be investigated systematically but a number of disparate observations are at least consistent with a close connection between oxidative stress and GS-X pump function: (i) All identified MRP-subclass transporters, including *AtMRP1* and *AtMRP2* recognize GSSG as a substrate. Studies of GS-X pumps originated from the discovery of ATP-dependent

GSSG efflux from erythrocytes (Srivastava and Beutler 1969, J. Biol. Chem. 244:9-16). (ii) In *S. cerevisiae*, overexpression of yAP1, a bZIP transcription factor, not only activates the YCF1 and GSH1 genes (Wemmie et al 1994, *supra*; Wu and Moye-Röwley 1994, *supra*), the latter of which encodes γ -glutamylcysteine synthetase, but also a panoply of oxidoreductases (DeRisi et al 1997, Science 278:680-686). Of the 17 genes whose mRNA levels are found to be increased by more than threefold on DNA microarrays by yAP1, more than two-thirds contain canonical upstream yAP1-binding sites (TTACTAA or TGACTAA), five bear homology to aryl-alcohol oxidoreductases and four to the general class of dehydrogenases/oxidoreductases (DeRisi et al 1997, *supra*). In view of the capacity of yAP1 overexpression to confer increased resistance to hydrogen peroxide, o-phenanthroline and heavy metals (Hirata et al 1994, Mol. Gen. Genet. 242:250-257), the fact that an appreciable fraction of the yAP1-regulated target genes identified against the yeast genome project database are oxidoreductases and coregulated with both YCF1 and GSH1, suggests that all of these genes play a protective role during oxidative stress. (iii) Two particularly harmful and early effects of AOS production are membrane lipid peroxidation and oxidative DNA damage which yield highly toxic 4-hydroxyalkenals (Esterbauer et al 1991, Biochem. J. 208:129-140) and base propanols (Berhane et al 1994, Proc. Natl. Acad. Sci. USA 91:1480-1484), respectively. Although such α,β -unsaturated aldehydes (and their GS-conjugates) have not yet been screened against the GS-X pumps from plant sources, they are established substrates for mammalian GSTs (Berhane et al 1994, *supra*) and their glutathionated derivatives are transported at high efficiency by mammalian GS-X pumps (Ishikawa 1989, J. Biol. Chem. 264:17343-17348).

There is therefore also included in the invention a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-X pump.

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of

illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

5

Experimental Examples

The experimental examples described herein provide procedures and results for the isolation and characterization of yeast *YCF1* and *Arabidopsis AtMRP1* and *AtMRP2* genes, gene products and various functions ascribed thereto. Further there is described data which establish that the *Bz2* gene product exerts its effects on plant coloration via the GS-X pump.

10

The data which are now described establish that *YCF1* is a vacuolar glutathione S-conjugate pump. The data establish that *YCF1* is a membrane protein which is responsible for catalyzing MgATP-dependent, uncoupler-insensitive uptake of glutathione S-conjugates into the vacuole of wild type *S. cerevisiae*.

15

YCF1 encodes a protein responsible for resistance of yeast to the effects of cadmium. However, the mechanism by which resistance to Cd^{2+} is effected was not understood until the present invention. The data presented herein demonstrate that *YCF1* confers Cd^{2+} resistance to yeast by effecting transport of Cd^{2+} out of the cytosol via a *YCF1* encoded vacuolar glutathione S-conjugate pump. Further, since *YCF1* confers resistance to Cd^{2+} through the transport of Cd.GS complexes or derivatives thereof, it is likely also capable of transporting other metal.GS-complexes. Examples of these other complexes include, but are not limited to, mercury (Hg), zinc (Zn), platinum (Pt) and arsenic (Ar). Both Hg^{2+} and Zn^{2+} form complexes with GSH which are analogous to those formed by Cd^{2+} (Li *et al.*, 1954, *J. Am. Chem. Soc.* 76:225-229; Kapoor *et al.*, 1965, *Biochem. Biophys. Acta* 100:376-383; Perrin *et al.*, 1971, *Biochem. Biophys. Acta* 230:96-104). In addition, MRP1 eliminates the Pt^{2+} glutathione complex bis(glutathionato)platinum from cancer cells (Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). Further, the MRP1 gene is overexpressed in cisplatin-resistant human leukemia HL-60 cells, which overexpression is associated

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with increased resistance to arsenite (Ishikawa *et al.*, 1996, *J. Biol. Chem.* 271:14981-14988). Both Hg^{2+} and As^{3+} are common environmental contaminants and Zn^{2+} is an essential micronutrient.

According to the results of the present study, vacuolar membrane vesicles from wild type *S. cerevisiae* catalyze high rates of MgATP-dependent, uncoupler-insensitive S-conjugate transport, and the kinetics of the transporter involved are similar to those of the mammalian and plant vacuolar GS-X pumps. In addition, vacuole-deficient mutants of *S. cerevisiae* exhibit markedly increased sensitivity to cadmium, leading to the belief that one requirement for efficient elimination or detoxification of this metal is maintenance of a sizable vacuolar compartment.

It is known that *S. cerevisiae* yAP-1 transcription factor transcriptionally activates both the *YCF1* gene and the *GSH1* gene (Wemmie *et al.*, 1994, *J. Biol. Chem.* 269:32592-32597; Wu *et al.*, 1994, *Mol. Cell. Biol.* 14:5832-5839). Since *GSH1* encodes γ -glutamylcysteine synthetase, an enzyme critical for GSH synthesis, expression of the *YCF1* gene and fabrication of one of the precursors for transport by the GS-X pump are coordinately regulated.

In the first set of experiments described below, transport of the model compounds DNP-GS and biman-GS by isolated membrane vesicles and intact cells was examined.

Yeast Strains and Plasmids

Two strains of *S. cerevisiae* were used in these studies: DTY165 (*MAT α ura3-52 his6 leu2-3,-112 his3- Δ 200 trp1-901 lys2-801 suc2- Δ*) and the isogenic *ycf1 Δ* mutant strain, DTY167 (*MAT α ura 3-52 his6 leu2-3,-112 his3- Δ 200 trp 1-901 lys2-801 suc2- Δ , *ycf1::hisG*). The strains were routinely grown in rich (YPD) medium, or, when transformed with plasmid containing functional *YCF1* gene, in synthetic complete medium (Sherman *et al.*, 1983, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, New York) or AHC medium (Kim *et al.*, 1994, *supra*) lacking the appropriate amino acids. *Escherichia coli* strains XL1-blue (Stratagene)*

and DH11S were employed for the construction and maintenance of plasmid stocks (Ausubel *et al.*, 1987, *Current Protocols in Molecular Biology*, Wiley, New York).

Plasmid pYCF1-HA, encoding epitope-tagged *YCF1*, was constructed in several steps. A 1.4-kb *SalI-HindIII* fragment, encompassing the carboxyl-terminal segment of the open reading frame of *YCF1*, from pIBIYCF1 (Szczypka *et al.*, 1994, *supra*), was subcloned into pBluescript KS⁻. Single-stranded DNA was prepared and used as template to insert DNA sequence encoding the human influenza hemagglutinin 12CA5 epitope immediately before the termination codon of the *YCF1* gene by oligonucleotide-directed mutagenesis. The sequence of the primer for this reaction, with the coding sequence for the 12CA5 epitope underlined, was 5'-
10 GTTTCACAGTTTAAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTG
ACC-3' (SEQ ID NO:12). After confirming the boundaries and fidelity of the HA-tag coding region by DNA sequencing, the 1.4-kb *SalI-HindIII* DNA fragment was exchanged with the corresponding wild type segment of pJAW50 (Wemmie *et al.*,
15 1994, *supra*) to generate pYCF1-HA.

Isolation of Vacuolar Membrane Vesicles

For the routine preparation of vacuolar membrane vesicles, 15 ml of stationary phase cultures of DTY165 or DTY167 were diluted into 1-liter volumes of fresh YPD medium, grown for 24 hours at 30°C to an OD_{600 nm} of approximately 0.8 and collected by centrifugation. After washing with distilled water, the cells were
20 converted to spheroplasts with Zymolyase 20T (ICN) (Kim *et al.*, 1994, *supra*) and intact vacuoles were isolated by flotation centrifugation of spheroplast lysates on Ficoll 400 step gradients as described by Roberts *et al.* (1991, *Methods. Enzymol.* 194:644-661). Both the spheroplast lysis buffer and Ficoll gradients contained 2
25 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF to minimize proteolysis. The resulting vacuole fraction was vesiculated in 5 mM MgCl₂, 25 mM KCl, 10 mM Tris-Mes (pH 6.9) containing 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF, pelleted by centrifugation at 37,000 x g for 25 min, and resuspended

in suspension medium (1.1 M glycerol, 2 mM dithiothreitol, 1 mM Tris-EGTA, 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, 5 mM Tris-Mes, pH 7.6) (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635).

5 In experiments involving cadmium transport, dithiothreitol and EGTA were removed from the suspension medium to prevent the attenuation of YCF1-dependent Cd^{2+} transport otherwise exerted by these compounds. Vesiculated vacuolar membranes were subjected to three cycles of 50-fold dilution into simplified suspension medium (1.1 M glycerol, 5 mM Tris-Mes, pH 8.0), centrifugation at
10 100,000 x g for 35 minutes and resuspension in the same medium before use.

For the experiment shown in Figure 4, 1 ml of partially purified vacuolar membrane vesicles (1.1-1.2 mg of protein), prepared by Ficoll flotation, were subjected to further fractionation by centrifugation through a 30-ml linear 10-40% (w/v) sucrose density gradient at 100,000 x g for 2 hours. Successive fractions were
15 collected from the top of the centrifuge tube and, after determining sucrose concentration refractometrically, the fractions were diluted with suspension medium. The diluted fractions were sedimented at 100,000 x g and resuspended in 100-µl aliquots of suspension medium for assay. For the immunoblots shown in Figure 5 and the marker enzyme analyses shown in Table 4, crude microsomes were prepared by
20 homogenization of spheroplasts in suspension medium and the sedimentation of total membranes at 100,000 x g for 35 minutes.

Microsomes and purified vacuolar membranes that were to be employed for SDS-polyacrylamide gel electrophoresis and immunoblotting were washed free of bovine serum albumin by three rounds of suspension in suspension
25 medium minus bovine serum albumin and centrifugation at 100,000 x g for 35 minutes. The final membrane preparations were either used immediately or frozen in liquid nitrogen and stored at -85°C.

Measurement of Marker Enzyme Activities

α -Mannosidase was determined according to Opheim (1978, *Biochem. Biophys. Acta* 524:121-125) using *p*-nitrophenyl- α -D-mannopyranoside as substrate. NADPH-cytochrome *c* reductase was estimated as FMN-promoted reduction of NADPH (Kubota *et al.*, 1977, *J. Biol. Chem.* 81:197-201). GDPase was measured as the rate of liberation of P_i from GDP (Yanagisawa *et al.*, 1990, *J. Biol. Chem.* 265:19351-19355) in reaction buffer containing 0.05% (w/v) Triton X-100. V-ATPase, F-ATPase, and P-ATPase were assayed as bafilomycin A_1 (1 μ M), azide (1 mM), and vanadate (100 μ M) inhibited ATPase activity, respectively, at pH 8.0 (V-ATPase, F-ATPase) or pH 6.5 (P-ATPase) (Rea and Turner, 1990, *Methods Plant Biochem.* 3:385-405).

Measurement of DNP-GS Uptake

Unless otherwise indicated, [3 H]DNP-GS uptake was measured at 25°C in 200 μ l reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 μ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0), and 66.2 μ M [3 H]DNP-GS (8.7 mCi/mmol) (Li *et al.*, 1995, *supra*). Gramicidin D was included in the uptake medium to abolish the H^+ electrochemical potential difference ($\Delta\mu_{H^+}$) that would otherwise be established by the V-ATPase in medium containing MgATP. Uptake was initiated by the addition of vacuolar membrane vesicles (10-15 μ g of membrane protein), brief mixing of the samples on a vortex mixer and uptake was then allowed to proceed for 1-60 minutes. Uptake was terminated by the addition of 1 ml of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter, 0.45 μ m). The filters were rinsed twice with 1 ml of ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in BCS mixture (Amersham Corp.). Nonenergized [3 H]DNP-GS uptake and extravesicular solution trapped on the filters were enumerated by the same procedure except that ATP and Mg^{2+} were omitted from the uptake medium.

Fluorescence Microscopy

Cells were grown in YPD medium for 24 hours at 30°C to an OD_{600 nm} of approximately 1.4, and 100 µl aliquots of the suspensions were transferred to 15 ml volumes of fresh YPD medium containing 100 µM *syn*-(ClCH₂,CH₃)-1,5-diazabicyclo-[3.3.0]-octa-3,6-dione-2,8-dione (monochlorobimane) (Kosower *et al.*, 1980, *J. Am. Chem. Soc.* 102:4983-4993). After incubation for 6 hours, the cells were pelleted by centrifugation, washed twice with YPD medium lacking monochlorobimane, and viewed without fixation under an Olympus BH-2 fluorescence microscope equipped with a BP-490 UV excitation filter, AFC-0515 barrier filter, and Nomarski optics attachment.

Electrophoresis and Immunoblotting

Membrane samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 7-12% (w/v) concave exponential gradient gels after delipidation with acetone:ethanol (Parry *et al.*, 1989, *J. Biol. Chem.* 264:20025-20032). The separated polypeptides were electrotransferred to 0.45 µm nitrocellulose filters at 60 V for 4 hours at 4°C in a Mini Trans-Blot transfer cell (Bio-Rad) and reversibly stained with Ponceau-S (Rea *et al.*, 1992, *Plant Physiol.* 100:723-732). The filters were blocked and incubated overnight with mouse anti-HA monoclonal antibody (20 µg/ml) (Boehringer-Mannheim). Immunoreactive bands were visualized by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (1/1000 dilution) (Boehringer-Mannheim) and incubation in buffer containing H₂O₂ (0.03% w/v), diaminobenzidine (0.6 mg/ml) and NiCl₂ (0.03% w/v) (Rea *et al.*, 1992, *supra*).

Purification of Cadmium-Glutathione Complexes

Singly radiolabeled ¹⁰⁹Cd.GS_n and doubly radio-labeled ¹⁰⁹Cd[³H].GS_n complexes were prepared by sequential gel-filtration and anion-exchange chromatography of the reaction products generated by incubating 20 mM ¹⁰⁹CdSO₄ (78.4 mCi/mmol) with 40 mM GSH or 40 mM [³H]GSH (240 mCi/mmol) in 15 ml 10 mM phosphate buffer (pH 8.0) containing 150 mM KNO₃ at 45°C for 24 hours. For gel-filtration, 2 ml aliquots of the reaction mixture were applied to a column (40 x 1.5 cm ID) packed with water-equilibrated Sephadex G-15, eluted with

deionized water and ^{109}Cd and/or ^3H in the fractions was measured by liquid scintillation counting. The fractions encompassed by each of the two $^{109}\text{Cd.GS}_n$ peaks identified were pooled, lyophilized and redissolved in 4 ml of loading buffer (5 mM Tris-Mes, pH 8.0). For anion-exchange chromatography, 0.5 ml aliquots of the resuspended lyophilizates from gel-filtration chromatography were applied to a Mono-Q HR5/5 column (Pharmacia) equilibrated with the same buffer. Elution was with a linear gradient of NaCl (0.5 ml/minute; 0-500 mM) dissolved in loading buffer. The individual fractions corresponding to the major peaks of ^{109}Cd obtained from the Mono-Q column (one each for the peaks resolved by gel-filtration chromatography) were pooled, lyophilized and resuspended in 4 ml deionized water after liquid scintillation counting. Buffer salts were removed before transport measurements or mass spectrometry by passing the samples down a column (120 x 1.0 cm ID) packed with water-equilibrated Sephadex G-15.

Measurement of $^{109}\text{Cd}^{2+}$ Uptake

MgATP-energized, uncoupler-insensitive $^{109}\text{Cd}^{2+}$ uptake by vacuolar membrane vesicles was measured at 25°C in 200 μl reaction volumes containing 3 mM ATP, 3 mM MgSO_4 , 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of $^{109}\text{CdSO}_4$, GSH or ^{109}Cd - and/or ^3H -labeled purified Cd.GS_n complexes as described herein except that the wash media contained 100 μM CdSO_4 in addition to sorbitol (400 mM) and Tris-Mes (3 mM, pH 8.0).

Pretreatment of DTY165 Cells with Cd^{2+} or 1-Chloro-2,4-dinitrobenzene

For studies on the inducibility of *YCF1* expression and YCF1-dependent transport, DTY165 cells were grown in YPD medium (Sherman *et al.*, 1983, *supra*) for 24 hours at 30°C to an $\text{OD}_{600\text{ nm}}$ of 1.0-1.2, pelleted by centrifugation and resuspended in fresh YPD medium containing CdSO_4 (200 μM) or 1-chloro-2,4-dinitrobenzene (CDNB). After washing in distilled water, total RNA was extracted and vacuolar membrane vesicles were prepared from the pretreated cells.

Control RNA and membrane samples were prepared from DTY165 cells treated in an identical manner except that CdSO_4 and CDNB were omitted from the second incubation cycle.

RNase Protection Assays

5 Cd^{2+} and CDNB-elicited increases in *YCF1* mRNA levels were assayed by RNase protection using 18S rRNA as an internal control. *YCF1*-specific probe was generated by PCR amplification of the full-length *YCF1::HA* gene, encoding human influenza hemagglutinin 12CA5 (HA) epitope-tagged *YCF1*, using plasmid pYCF1-HA as template. The forward *YCF1*-specific primer and backward primer containing
10 the HA-tag coding sequence had the sequences 5'-AAACTGCAGATGGCTGGTAATCTTGTTTC-3' (SEQ ID NO:13) and 5'-GCCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTGA-3' (SEQ ID NO:14), respectively. An 18S rRNA-specific probe was synthesized by PCR of *S. cerevisiae* genomic DNA using sense and antisense primers having the sequences
15 5'-AGATTAAGCCATGCATGTCT-3' (SEQ ID NO:15) and 5'-TGCTGGTACCAGACTTGCCCTCC-3' (SEQ ID NO:16), respectively. Both PCR products were individually subcloned into pCRTMII vector (Invitrogen) to generate plasmids pCR-*YCF1* and pCR-Y18S. After linearization of pCR-*YCF1* and pCR-Y18S with *Afl*I and *Nco*I, a 320-nucleotide *YCF1*-specific RNA probe and 220-
20 nucleotide 18S rRNA-specific probe were synthesized using T7 RNA polymerase and SP6 RNA polymerase, respectively. Aliquots of total RNA, prepared as described (Kohrer *et al.*, 1991, *Methods in Enzymol.* 194:390-398), from control, CdSO_4 - or CDNB-pretreated DTY165 cells were hybridized with a mixture of ^{32}P -labeled *YCF1* antisense probe (1×10^6 cpm) and 18S rRNA antisense probe (5×10^2 cpm) and
25 RNase protection (Teeter *et al.*, 1990, *Mol. Cell. Biol.* 10:5728-5735) was assayed using an RPAII kit (Ambion).

Matrix-Assisted Laser Desorption Mass Spectrometry (MALD-MS)

The $^{109}\text{Cd}.\text{GS}_n$ complexes purified by gel-filtration and anion-exchange chromatography were adjusted to a final concentration of 2-5 mM (as Cd)

with deionized water, mixed with an equal volume of sinapinic acid (10 mg/ml) dissolved in acetonitrile/H₂O/trifluoroacetic acid (70:30:0.1 % (v/v)) and applied to the ion source of a PerSeptive Biosystems Voyager RP Biospectrometry Workstation. The instrument, which was equipped with a 1.3 m flight tube and variable two-stage ion source set at 30 kV, was operated in linear mode. Mass/charge (m/z) ratio was measured by time-of-flight after calibration with external standards.

Protein Estimations

Protein was estimated by a modification of the method of Peterson (1977, *Anal. Biochem.* 83:346-356).

Chemicals

S-(2,4-dinitrophenyl)glutathione (DNP-GS) was synthesized from 1-chloro-2,4-dinitrobenzene (CDNB) and GSH by the procedure of Kunst *et al.* (1983, *Biochem. Biophys. Acta* 983:123-125) and (Li *et al.*, 1995, *supra*). [³H]DNP-GS (specific activity, 8.7 mCi/mmol) and bimane-GS were synthesized enzymatically and purified by a modification of the procedure of Kunst *et al.* (1983, *supra*) according to Li *et al.* (1995, *supra*). Metolachlor-GS was synthesized by general base catalysis and purified by reverse-phase high performance liquid chromatography (Li *et al.*, 1995, *supra*).

GSH and CDNB were purchased from Fluka; AMP-PNP, aprotinin, ATP, creatine kinase (type I from rabbit muscle, 150-250 units/mg of protein), creatine phosphate, FCCP, oxidized glutathione (GSSG), S-methylglutathione, cysteinylglycine, cysteine, glutamate and gramicidin D, leupeptin, PMSF, verapamil, and vinblastine were from Sigma; monochlorobimane was from Molecular Probes; cellulose nitrate membranes (0.45- μ m pore size, HA filters) were from Millipore; [³H]glutathione[(glycine-2-³H]-L-Glu-Cys-Gly; 44 Ci/mmol) was from DuPont NEN; and ¹⁰⁹CdSO₄ (78.44 Ci/mmol) was from Amersham Corp. Metolachlor was a gift from CIBA-Geigy, Greensboro, NC. All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

Sensitivity to CDNB

If the *YCF1* gene product were to participate in the detoxification of *S*-conjugable xenobiotics, mutants deleted for this gene would be expected to be more sensitive to the toxic effects of these compounds than wild type cells. This is what was found (Figure 1).

5 The isogenic wild type strain DTY165 and the *ycf1*Δ mutant strain, DTY167, were indistinguishable during growth in YPD medium lacking CDNB; both strains grew at the same rate after a brief lag. However, the addition of CDNB to the culture medium caused a greater retardation of the growth of DTY167 cells (Figure 1B) than DTY165 cells (Figure 1A). Inhibitory concentrations of CDNB resulted in a
10 slower, more linear, growth rate for at least 24 hours for both strains, but DTY167 underwent growth retardation at lower concentrations than did DTY165. The optical densities of the DTY167 cultures were diminished by 65, 82, 85, and 91% by 40, 50, 60, and 70 μM CDNB, respectively, after 24 hours of incubation (Figure 1B), whereas the corresponding diminutions for the DTY165 cultures were 14, 31, 59, and 92%
15 (Figure 1A). The increase in sensitivity to CDNB conferred by deletion of the *YCF1* gene was similar to that seen with cadmium.

Impaired Vacuolar DNP-GS Transport

Vacuolar membrane vesicles purified from DTY165 cells exhibited high rates of MgATP-dependent [³H]DNP-GS uptake (Figure 2). Providing that
20 creatine phosphate and creatine kinase were included in the uptake media to ensure ATP regeneration, addition of 3 mM MgATP increased the initial rate of DNP-GS uptake by 122-fold to a value of 12.2 nmol/mg/minute. The same membrane fraction from DTY167 cells, although capable of similar rates of MgATP-independent DNP-GS uptake, was only 17-fold stimulated by MgATP and capable of an initial rate of
25 uptake of only 1.7 nmol/mg/minute (Figure 2).

Selective Impairment of Uncoupler-Insensitive Transport

Direct comparisons between vacuolar membrane vesicles from DTY165 and DTY167 cells demonstrated that deletion of the *YCF1* gene selectively abolished MgATP-energized, Δμ_H⁺-independent DNP-GS transport.

Agents that dissipate both the pH (ΔpH) and electrical ($\Delta\psi$) components of the $\Delta\mu_{\text{H}^+}$ established by the V-ATPase (FCCP, gramicidin D) or directly inhibit the V-ATPase, itself (bafilomycin A_1), decreased MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles from DTY165 cells from 77.7 ± 1.0 nmol/mg/10 minutes to between 43.2 ± 1.0 and 47.4 ± 1.7 nmol/mg/10 minutes (Table 1). Ammonium chloride, which abolishes ΔpH while leaving $\Delta\psi$ unaffected, on the other hand, did not inhibit DNP-GS uptake (Table 1). On the basis of these characteristics, the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone, and the resistance of 50-60% of total uptake to inhibition by any one of these compounds (Table 1), DNP-GS uptake by vacuolar membranes from wild type cells is concluded to proceed via two parallel mechanisms: a V-ATPase inhibitor- and uncoupler-insensitive pathway that is directly energized by MgATP, and a $\Delta\mu_{\text{H}^+}$ -dependent, V-ATPase inhibitor-sensitive and uncoupler-sensitive pathway that is primarily driven by the inside-positive $\Delta\psi$ established by the V-ATPase.

Of these two pathways, the $\Delta\psi$ -dependent pathway predominated in membranes from DTY167 cells (Table 1). FCCP, gramicidin D, and bafilomycin A_1 diminished net DNP-GS uptake by DTY167 vacuolar membranes from 15.4 ± 0.4 nmol/mg/10 minutes to between 4.3 ± 0.3 and 6.4 ± 0.3 nmol/mg/10 minutes. Moreover, although the effects of FCCP or gramicidin D and V-ATPase inhibitors in combination were slightly greater than those seen when these agents were added individually, the transport remaining was only about 10% of that seen with wild type membranes and only 2-4-fold stimulated by MgATP. In conjunction with the negligible inhibitions seen with NH_4Cl , alone, indicating that $\Delta\psi$, not ΔpH , is the principal driving force for the transport activity remaining in their vacuolar membranes, DTY167 cells are inferred to be preferentially impaired in MgATP-energized, $\Delta\mu_{\text{H}^+}$ -independent DNP-GS transport.

The nonhydrolyzable ATP analog, AMP-PNP, did not promote DNP-GS uptake by vacuolar membrane vesicles from either DTY165 or DTY167 cells

(Table 1), indicating a requirement for hydrolysis of the γ -phosphate of ATP regardless of whether uptake was via the YCF1- or $\Delta\psi$ -dependent pathway.

Table 1. Effects of MgATP, MgAMP-PNP, protonophores, ionophores and V-ATPase inhibitors on $[^3\text{H}]\text{DNP-GS}$ uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.		
Uptake was measured for 10 minutes in standard uptake medium described herein containing 66.2 μM $[^3\text{H}]\text{DNP-GS}$ plus the compounds indicated. MgATP (3 mM) was present throughout unless otherwise indicated. MgAMP-PNP, bafilomycin A_1 , FCCP, gramicidin D, and NH_4Cl were added at concentrations of 3 mM, 0.5 μM , 5 μM , 5 μM and 1 mM, respectively. Values outside parentheses are means \pm SE ($n = 3-6$); values inside parentheses are rates of uptake expressed as percentage of control.		
ADDITIONS	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	77.7 \pm 1.0 (100)	15.4 \pm 0.4 (100)
-MgATP	2.2 \pm 0.4 (2.8)	1.5 \pm 0.6 (9.7)
MgAMP-PNP(-MgATP)	2.5 \pm 0.5 (3.2)	1.4 \pm 0.3 (9.1)
FCCP	47.4 \pm 1.7 (61.0)	6.4 \pm 0.3 (41.8)
Gramicidin D	45.8 \pm 1.4 (58.9)	5.8 \pm 0.1 (37.7)
NH_4Cl	69.1 \pm 2.9 (88.9)	14.9 \pm 0.7 (96.8)
NH_4Cl + gramicidin D	42.6 \pm 1.8 (54.8)	4.1 \pm 0.2 (26.6)
Bafilomycin A_1	43.2 \pm 1.0 (55.6)	4.3 \pm 0.3 (27.9)
Bafilomycin A_1 + gramicidin D	39.2 \pm 2.6 (50.5)	3.8 \pm 0.1 (24.7)

Abolition of High Affinity, Uncoupler-insensitive Uptake

Examination of the concentration dependence of $[^3\text{H}]\text{DNP-GS}$ uptake revealed a near total abolition of high affinity, MgATP-dependent, uncoupler-insensitive transport by vacuolar membrane vesicles from the *ycf1* Δ mutant strain (Figure 3). When measured in the presence of uncoupler (gramicidin D), the rate of DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells increased as a simple hyperbolic function of MgATP (Figure 3A) and DNP-GS concentration (Figure 3B) to yield K_m values OF 86.5 \pm 29.5 μM (MgATP) and 14.1 \pm 7.4 μM

(DNP-GS) and a V_{\max} of 51.0 ± 6.3 nmol/mg/10 minutes (DNP-GS). By contrast, uncoupler-insensitive uptake by the corresponding membrane fraction from DTY167 cells was more than 15-fold slower over the entire concentration range, showed no evidence of saturation and increased as a linear function of both DNP-GS and MgATP concentration (Figure 3).

Selective Inhibitors of YCF1-mediated Transport

MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells was sensitive to inhibition by vanadate, vinblastine, verapamil, GSSG and glutathione *S*-conjugates other than DNP-GS (Tables 2 and 3). One hundred μ M concentrations of metolachlor-GS, azidophenacyl-GS and bimeane-GS and 1 mM GSSG inhibited uptake by about 50% (Table 2), while vanadate, vinblastine, and verapamil exerted 50% inhibitions at concentrations of 179, 89 and 203 μ M, respectively (Table 3). None of these agents significantly inhibited residual MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles from DTY167 cells (Tables 2 and 3).

TABLE 2. Effects of GSH, GSSG, and glutathione S-conjugates other than DNP-GS on MgATP-dependent, uncoupler-insensitive [3 H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured as described for Table 1 except that 5 μ M gramicidin D was included in all of the uptake media. Values outside parentheses are means \pm SE ($n = 3-6$); values inside parentheses are rates of uptake expressed as percentage of control.

COMPOUND	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	47.9 \pm 2.5 (100)	6.5 \pm 0.8 (100)
GSH (1 mM)	50.6 \pm 2.3 (105.6)	4.6 \pm 1.1 (70.8)
GSSG (1 mM)	26.0 \pm 0.9 (54.3)	4.4 \pm 0.4 (67.7)
Metolachlor-GS (100 μ M)	27.6 \pm 0.9 (57.7)	4.8 \pm 0.7 (73.8)
Azidophenacyl-GS (100 μ M)	16.0 \pm 1.4 (33.5)	5.2 \pm 0.3 (80.0)
Bimane-GS (100 μ M)	25.2 \pm 1.1 (52.6)	4.5 \pm 0.4 (69.2)

TABLE 3. Sensitivity of MgATP-dependent, uncoupler-insensitive [3 H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells to inhibition by vanadate, vinblastine, and verapamil. Uptake was measured as described in Table 1 except that 5 μ M gramicidin D was included in all of the uptake media. The concentrations of the compounds causing 50% inhibition of uptake (I_{50} values) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential (Marquardt, 1963, *supra*).

Addition	I_{50}	
	DTY165	DTY167
	μ M	
Vanadate	179.1	Insensitive
Vinblastine	88.8	> 500
Verapamil	202.6	Insensitive

Vacuolar Membrane Localization

The capacity for MgATP-dependent, uncoupler-insensitive [3 H]DNP-GS uptake strictly copurified with the vacuolar membrane fraction (Table 4). By

comparison with crude microsomes (total membranes) prepared from whole spheroplast homogenates of DTY165 cells, vacuolar membrane vesicles derived from vacuoles purified by the Ficoll flotation technique were coordinately enriched for DNP-GS uptake and for both of the vacuolar membrane markers assayed, α -mannosidase and bafilomycin A₁-sensitive ATPase (V-ATPase) activity. The respective enrichments of MgATP-dependent, uncoupler-insensitive DNP-GS uptake, α -mannosidase and bafilomycin A₁-sensitive ATPase activity were 28-, 53- and 22-fold. By contrast, the vacuolar membrane fraction was 4.5-, 6.3-, 11.1- and 4.3-fold depleted of NADPH cytochrome *c* reductase (endoplasmic reticulum), latent GDPase (Golgi), vanadate-sensitive ATPase (plasma membrane), and azide-sensitive ATPase activity (mitochondrial inner membrane), respectively. Accordingly, when vacuolar membrane vesicles derived from Ficoll-flotated vacuoles were subjected to further fractionation on linear 10–40% (w/v) sucrose density gradients, MgATP-dependent, uncoupler-insensitive [³H]DNP-GS uptake, α -mannosidase and bafilomycin A₁-sensitive ATPase activity were found to comigrate and exhibit identical density profiles (Figure 4).

TABLE 4. Comparison of rates of MgATP-dependent, uncoupler-insensitive [³ H]DNP-GS transport and specific activities of marker enzymes in crude microsomes and vacuolar membrane vesicles prepared from DTY165 cells. Microsomes and vacuolar membrane vesicles were prepared from spheroplasts and the marker enzymes were assayed as described herein. Values shown are means \pm SE ($n = 3$).			
PREPARATION	ACTIVITY		
	DNP-GS UPTAKE	α -mannosidase	NADPH-cyt c reductase
	nmol/mg/10 min	nmol/mg/min	nmol/mg/min
Microsomes	2.5 \pm 0.3	6.3 \pm 0.3	88.0 \pm 1.3
Vacuolar membrane	69.9 \pm 1.0	329.3 \pm 3.2	19.3 \pm 0.6
Enrichment (-fold)	27.96	52.27	0.22

PREPARATION	ACTIVITY			
	V-ATPase	GDPase	P-ATPase	F-ATPase
	μ mol/mg/h		μ mol/mg/h	
Microsomes	11.7 \pm 6.3	35.0 \pm 1.1	37.1 \pm 4.6	155.6 \pm 3.0
Vacuolar membrane	253.1 \pm 15.8	5.5 \pm 0.1	3.2 \pm 1.6	35.1 \pm 8.4
Enrichment (-fold)	21.63	0.16	0.09	0.23

Plasmid-encoded YCF1 Mediates Vacuolar DNP-GS Transport and

CDNB Resistance

5 Immunoblots of vacuolar membranes from pYCF1-HA-transformed DTY165 or DTY167 cells, probed with mouse anti-HA monoclonal antibody, demonstrated incorporation of YCF1-HA polypeptide into the vacuolar membrane fraction (Figure 5B). Immunoreaction with the 12CA5 epitope was not detectable in lanes loaded with membranes from pRS424-transformed cells but the same quantities of membranes prepared from pYCF1-HA-transformed cells yielded a single intensely immunoreactive band with an electrophoretic mobility ($M_r = 156,200$) commensurate

with a computed mass of 172 kDa for the fusion protein encoded by YCF1-HA (Figure 5B).

Direct participation of the plasmid-borne YCF1-HA gene product in DNP-GS transport and CDNB detoxification was verified by the finding that vacuolar membrane vesicles purified from pYCF1-HA-transformed DTY167 cells exhibited a 6-fold enhancement of MgATP-dependent, uncoupler-insensitive [3 H]DNP-GS uptake (Figure 5A) which was accompanied by a decrease in the susceptibility of such transformants to growth retardation by exogenous CDNB (Figure 6). Whereas pYCF1-HA-transformed DTY167 cells exhibited a similar resistance to growth retardation by CDNB as untransformed DTY165 cells (compare Figure 6B with Figure 1A), the same mutant strain showed neither increased vacuolar DNP-GS transport *in vitro* nor decreased susceptibility to CDNB *in vivo* after transformation with parental plasmid pRS424, lacking the YCF1-HA insert (Figure 6B).

Vacuolar Accumulation of Bimane-GS *In Vivo*

Monochlorobimane, a membrane-permeant, nonfluorescent compound, is specifically conjugated with GSH by cytosolic glutathione *S*-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant *S*-conjugate, bimane-GS (Shrieve *et al.*, 1988, *J. Biol. Chem.* 263:14107-12114; Oude Elferink *et al.*, 1993, *Hepatology* 17:343-444; Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). The GS-X pumps of both animal and plant cells exhibit activity toward a broad range of *S*-conjugates, including bimane-GS (Ishikawa *et al.*, 1994, *supra*; Martinoia *et al.*, 1993, *supra*), and DNP-GS uptake by the yeast enzyme is shown herein to be reversibly inhibited by this compound (Table 2). These data suggest competition between bimane-GS and DNP-GS for a common uptake mechanism. Exogenous monochlorobimane therefore satisfies the minimum requirements of a sensitive probe for monitoring the intracellular transport and localization of its *S*-conjugate.

Fluorescence microscopy of DTY165 and DTY167 cells after incubation in growth medium containing monochlorobimane provides direct evidence that YCF1 contributes to the vacuolar accumulation of its glutathione *S*-conjugate by

intact cells (Figure 7). DTY165 cells exhibited an intense punctate fluorescence, corresponding to the vacuole as determined by Nomarski microscopy, after 6 hours of incubation with monochlorobimane (Figures 7A and 7C). The fluorescence associated with vacuolar bimane-GS was by comparison severely attenuated in most, and completely absent from many, DTY167 cells (Figures 7B and 7D).

*ycf1*Δ Mutants are Defective in GSH-Dependent Cd²⁺ Transport

Physiological (1 mM) concentrations of GSH (Kang, 1992, *Drug Metabolism and Disposition* 20:714-718) promoted Cd²⁺ uptake by vacuolar membrane vesicles purified from the wild type strain DTY165 but not the *ycf1*Δ mutant strain DTY167 (Figure 8). Addition of Cd²⁺ (80 μM) to GSH-containing media elicited MgATP-dependent, uncoupler-insensitive ¹⁰⁹Cd²⁺ uptake rates of 4.5 and 0.8 nmol/mg/minute by DTY165 and DTY167 membranes, respectively (Figures 8A and 8B). Uptake by DTY165 membranes was diminished more than 9-fold by the omission of GSH (Figure 8A) whereas uptake by DTY167 membranes was slightly stimulated (Figure 8B).

GSH maximally stimulated uptake within minutes ($t_{1/2} < 5$ minutes) of the addition of Cd²⁺ to the uptake medium and uptake was sigmoidally dependent on Cd²⁺ concentration, achieving half-maximal velocity at 120 μM (Figure 8C).

Specific Requirement for GSH

The stimulatory action of GSH was abolished by the omission of MgATP from the assay medium (Figure 8 and Table 5) and 1 mM concentrations of GSSG, S-methylglutathione, cysteinylglycine, cysteine or glutamate did not promote MgATP-dependent, uncoupler-insensitive Cd²⁺ uptake by vacuolar membrane vesicles from either strain (Table 5).

TABLE 5. Effects of different GSH-related compounds on uncoupler-insensitive ^{109}Cd uptake by vacuolar membrane vesicles purified from DTY165 or DTY167 cells. GSH, oxidized glutathione (GSSG), S-methylglutathione (GS-CH₃), cysteinylglycine, cysteine and glutamate were added at concentrations of 1 mM. MgATP, $^{109}\text{CdSO}_4$ and gramicidin-D were added at concentrations of 3 mM, 80 μM and 5 μM , respectively. Values shown are means \pm SE ($n = 3-6$).

COMPOUND	^{109}Cd UPTAKE (nmol/mg/10 minutes)			
	DTY165		DTY167	
	-MgATP	+MgATP	-MgATP	+MgATP
Cd^{2+}	5.8 \pm 2.4	5.6 \pm 1.5	4.3 \pm 1.3	4.6 \pm 2.1
Cd^{2+} + GSH	4.2 \pm 1.2	37.4 \pm 4.5	3.3 \pm 1.1	8.3 \pm 2.7
Cd^{2+} + GSSG	-	5.1 \pm 3.2	-	3.8 \pm 2.3
Cd^{2+} + GS-CH ₃	-	4.5 \pm 1.9	-	3.7 \pm 3.1
Cd^{2+} + Cys-Gly	-	5.6 \pm 3.2	-	6.9 \pm 1.4
Cd^{2+} + Cys	-	7.0 \pm 1.2	-	3.9 \pm 1.0
Cd^{2+} + Glu	-	5.7 \pm 1.1	-	5.2 \pm 1.3

Purification of Transport-Active Complex

To determine the mode of action of GSH and the form in which Cd^{2+} is transported, reaction mixtures initially containing Cd^{2+} and GSH were fractionated and *YCF1*-dependent uptake was assayed.

5 Incubation of $^{109}\text{Cd}^{2+}$ with GSH and gel-filtration of the mixture on Sephadex G-15 yielded two major ^{109}Cd -labeled peaks: a low molecular weight peak (*LMW-Cd.GS*) and a high molecular weight peak (*HMW-Cd.GS*) (Figure 9A). When rechromatographed on Mono-Q, *LMW-Cd.GS* and *HMW-Cd.GS* eluted at 0 (Figure 9C) and 275 mM NaCl, respectively (Figure 9B). Of these two ^{109}Cd -labeled components,

10 *HMW-Cd.GS* alone, underwent *YCF1*-dependent transport. MgATP-dependent, uncoupler-insensitive *HMW- $^{109}\text{Cd.GS}$* uptake by DTY165 membranes increased as a single Michaelian function of concentration (K_m , 39.1 \pm 14.1 μM ; V_{max} , 157.2 \pm 60.7 nmol/mg/10 minutes) (Figure 10A). By contrast, uptake of *LMW- $^{109}\text{Cd.GS}$* by DTY165 membranes was negligible at all of the concentrations examined (Figure

15 10B). Vacuolar membranes from DTY167 cells transported neither *HMW- $^{109}\text{Cd.GS}$* nor *LMW- $^{109}\text{Cd.GS}$* (Figures 10A and 10B).

Bis(glutathionato)cadmium Is the Transport-Active Complex

The transport-active complex, *HMW-Cd.GS*, was identified as *bis*(glutathionato)cadmium (Cd.GS_2) by three criteria: (i) The average Cd:GS molar ratio of the transported species, estimated from the $^{109}\text{Cd}:$ ^3H ratios of the *HMW-Cd.GS* peaks obtained after chromatography of reaction mixtures initially containing $^{109}\text{Cd}^{2+}$ and ^3H GSH on Sephadex G-15 and Mono-Q were 0.44 ± 0.09 and 0.49 ± 0.17 , respectively (Table 6). (ii) DTY165 membranes accumulated ^{109}Cd and ^3H GS in a molar ratio of 0.49 ± 0.01 when incubated in media containing *HMW- $^{109}\text{Cd}.$ ^3H GS*, MgATP and gramicidin-D (Table 6). (iii) The principal ion peak detected after MALD-MS of *HMW-Cd.GS* had an m/z ratio of 725.4 ± 0.7 , consistent with the molecular weight of *bis*(glutathionato)cadmium (724.6 Da, Figure 11). The transport-inactive complex, *LMW-Cd.GS*, on the other hand, was tentatively identified as *mono*(glutathionato)cadmium on the basis of its smaller apparent molecular size (Figure 9A), failure to bind Mono-Q (Figure 9C) and Cd:GS ratio of 0.67 ± 0.04 and 0.86 ± 0.07 after chromatography on Sephadex G-15 and Mono-Q (Table 6), respectively.

While an m/z ratio of 725 for *HMW-Cd.GS* would be equally compatible with the transport of Cd.GSSG , this is refuted by two findings: (i) GSSG alone does not promote *YCF1*-dependent uptake (Table 5). (ii) The transport-active complex is probably a mercaptide. Pretreatment of *HMW-Cd.GS* with 2-mercaptoethanol inhibits MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by DTY165 membranes by more than 80% (Table 6) and *S*-methylation abolishes the stimulatory action of GSH (Table 5).

TABLE 6. Molar Cd:GS ratios of LMW-Cd:GS and HMW-Cd:GS complexes fractionated by Sephadex G-15 and Mono-Q chromatography (Figure 9) before and after MgATP-dependent, uncoupler-insensitive uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Cd:GS ratios were estimated from the $^{109}\text{Cd}:[^3\text{H}]$ radioisotope ratios of samples prepared from $^{109}\text{CdSO}_4$ and $[^3\text{H}]\text{GSH}$. HMW- $^{109}\text{Cd}:[^3\text{H}]\text{GS}$ was pretreated with 2-mercaptoethanol (2-ME) by heating a 1:4 mixture of HMW- $^{109}\text{Cd}:[^3\text{H}]\text{GS}$ with 2-ME at 60°C for 10 minutes before measuring $^{109}\text{Cd}^{2+}$ uptake. Uptake was measured using $50\text{ }\mu\text{M}$ concentrations (as Cd) of the complexes indicated in standard uptake medium containing $5\text{ }\mu\text{M}$ gramicidin-D. Values shown are means \pm SE ($n = 3-6$).

FRACTION	^{109}Cd UPTAKE (nmol/mg/10 min)		MOLAR RATIO Cd:GS	
	DTY165	DTY167	BEFORE UPTAKE	AFTER UPTAKE
Sephadex G-15				
HMW-Cd:GS	-	-	0.44 ± 0.09	-
LMW-Cd:GS	-	-	0.67 ± 0.04	-
Mono-Q				
HMW-Cd:GS	66.3 ± 2.7	5.6 ± 2.6	0.49 ± 0.17	0.49 ± 0.01
LMW-Cd:GS	4.4 ± 0.8	3.9 ± 1.4	0.86 ± 0.07	-
After 2-ME				
HMW-Cd:GS	11.9 ± 2.4	4.4 ± 3.0	-	-

Cd:GS₂ Transport is Directly Energized by MgATP

Purification of Cd:GS₂ enabled the energy requirements of YCF1-dependent transport to be examined directly and confirmed that more than 83% of the MgATP-dependent, uncoupler-insensitive Cd²⁺ transport measured using DTY165 membranes was mediated by YCF1. Agents that dissipate both the ΔpH and $\Delta\psi$ components of the H⁺-electrochemical gradient established by the V-ATPase (FCCP, gramicidin-D) or directly inhibit the V-ATPase, itself (bafilomycin A₁), decreased MgATP-dependent Cd:GS₂ uptake by vacuolar membrane vesicles from DTY165 cells by 22% (Table 7). Ammonium chloride which abolishes ΔpH while leaving $\Delta\psi$ unaffected, on the other hand, inhibited uptake by only 15% (Table 7). From these results and the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors alone (Table 7), Cd:GS₂ uptake by wild type membranes is

inferred to proceed *via* a YCF1-dependent, MgATP-energized pathway that accounts for most of the transport measured and a YCF1-independent pathway, primarily driven by the H^+ -gradient established by the V-ATPase, that makes a minor contribution to total uptake.

Table 7. Effects of uncouplers and V-ATPase inhibitors on uptake of bis(glutathionato)cadmium ($Cd.GS_2$) by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 50 μM purified $^{109}Cd.GS_2$. Bafilomycin A_1 , FCCP, gramicidin-D and NH_4Cl were added at concentrations of 0.5 μM , 5 μM , 5 μM , and 1 mM, respectively. Values outside parentheses are means \pm SE ($n = 3-6$); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITION	$^{109}Cd.GS_2$ UPTAKE (nmol/mg/10 minutes)	
	DTY165	DTY167
Control	105.8 \pm 12.4 (100)	17.3 \pm 2.7 (100)
Gramicidin-D	77.8 \pm 6.4 (73.5)	9.3 \pm 2.0 (56.6)
FCCP	62.2 \pm 11.4 (58.8)	10.2 \pm 1.6 (59.0)
NH_4Cl	89.8 \pm 8.2 (84.8)	10.0 \pm 1.7 (57.8)
NH_4Cl + gramicidin-D	69.8 \pm 12.0 (66.0)	8.8 \pm 2.2 (50.9)
Bafilomycin A_1	81.8 \pm 6.0 (76.6)	12.8 \pm 3.6 (74.0)
Bafilomycin A_1 + gramicidin-D	70.2 \pm 12.2 (66.4)	7.2 \pm 2.4 (41.6)

$Cd.GS_2$ Competes with DNP-GS for Transport

As would be predicted if $Cd.GS_2$ and the model organic GS-conjugate DNP-GS follow the same transport pathway, the K_i for inhibition of MgATP-dependent, uncoupler-insensitive $Cd.GS_2$ uptake by DNP-GS ($11.3 \pm 2.1 \mu M$; Figures 10A and 10C) coincided with the K_m for DNP-GS transport ($14.1 \pm 7.4 \mu M$).

Pretreatment with Cd^{2+} or CDNB Increases YCF1 Expression

RNase protection assays of YCF1 expression in DTY165 cells and measurements of MgATP-dependent, uncoupler-insensitive $^{109}Cd.GS_2$ and $[^3H]DNP-GS$ uptake by vacuolar membranes prepared from the same cells after 24 hour of growth in media containing $CdSO_4$ (200 μM) or the cytotoxic DNP-GS precursor, CDNB (150 μM), demonstrated a parallel increase in all three quantities. YCF1-

specific mRNA levels were increased by 1.9- and 2.5-fold by pretreatment of DTY165 cells with CdSO_4 and CDNB, respectively (Figure 12). The same pretreatments increased MgATP-dependent, uncoupler-insensitive $^{109}\text{Cd.GS}_2$ uptake into vacuolar membrane vesicles by 1.4- and 1.7-fold and $[^3\text{H}]\text{DNP-GS}$ uptake by 1.6- and 2.8-fold (Figure 12).

These investigations provide the first indication of the mechanism by which *YCF1* confers Cd^{2+} resistance in *S. cerevisiae* and its relationship to the transport of organic GS-conjugates by demonstrating that the integral membrane protein encoded by this gene specifically catalyzes the MgATP-energized uptake of bis(glutathionato)cadmium by vacuolar membrane vesicles.

The codependence of Cd.GS_2 and organic GS-conjugate transport on *YCF1* is evident at multiple levels: (i) The *ycf1* Δ mutant strain, DTY167, is hypersensitive to Cd^{2+} and CDNB in the growth medium and both hypersensitivities are alleviated by transformation with plasmid-borne *YCF1*. (ii) Vacuolar membrane vesicles purified from DTY167 cells are grossly impaired for MgATP-energized, uncoupler-insensitive organic GS-conjugate and GSH-promoted Cd^{2+} uptake. (iii) Cd.GS_2 and organic GS-conjugates compete for the same uptake sites on *YCF1*. (iv) Factors that increase *YCF1* expression elicit a parallel increase in Cd.GS_2 and organic GS-conjugate transport. Thus, a number of ostensibly disparate observations, the strong association between cellular GSH levels and Cd^{2+} resistance (e.g., Singhal *et al.*, 1987, *FASEB J.* 1:220-223), the markedly increased sensitivity of vacuole deficient *S. cerevisiae* strains to Cd^{2+} toxicity, and the coordinate regulation of the yeast *YCF1* and *GSH1* genes, the latter of which encodes γ -glutamylcysteine synthetase (Wemmie *et al.*, 1994, *supra*; Wu *et al.*, 1994, *supra*), are now explicable in terms of a model in which *YCF1* catalyzes the GSH-dependent vacuolar sequestration of Cd^{2+} .

Further, at the biochemical level, *YCF1* specifically catalyzes the transport of Cd.GS_2 as the data provided herein establish. In addition, at the cellular level, *YCF1* confers resistance to and is induced by a spectrum of xenobiotics. Expression of *YCF1* is increased by exposure of cells to glutathione-conjugable

5 xenobiotics and Cd^{2+} . The close resemblance of YCF1 to MRP1, the capacity of YCF1 for both organic toxin and heavy metal transport, and its discovery in one of the most tractable and thoroughly molecularly characterized eukaryotes, *S. cerevisiae*, establishes that YCF1 is useful for manipulation of the transport of organic toxins and heavy metals in plants, mammals and yeast. Thus, according to the present invention, methods for overcoming, or at least diminishing, heavy metal contamination through bioremediation using native species or genetically engineered organisms are now possible.

Cloning of plant MRP1/YCF1 homologs

10 As described herein, the data presented herein establish that YCF1 encodes a protein functionally equivalent to human MRP1. There is next described the discovery that two plant genes, *AtMRP1* and *AtMRP2*, from *Arabidopsis* encode MRP1/YCF1 homologs.

15 To isolate genes likely involved in glutathione S-conjugate transport from *Arabidopsis thaliana*, degenerate PCR primers corresponding to appropriate portions of human MRP1 (Cole *et al.*, 1992, *supra*) and YCF1 (Szczypka *et al.*, 1994, *supra*) were designed. Four degenerate primers were synthesized but only two of these yielded amplification products of the appropriate size that hybridized with MRP1 and YCF1. The sequences of the two primers were:

20 5'-GARAARGTIGGIATHGTIGGIMGIACIGGIGC-3'(MRP2) (SEQ ID NO:17) and 5'-TCCATDATIGTRTTIARICKTGIGC-3'(MRP4) (SEQ ID NO:18), where I = inosine, K = T or G, M = C or A and R = A or G. MRP2 corresponds to positions 1321-1331 and 1300-1310 in MRP1 and YCF1, respectively; MRP4 corresponds to positions 1486-1494 and 1466-1474. Database searches confirmed that the sequences

25 of the peptides specified by MRP2 and MRP4 were specific to MRP1 and YCF1 but not any other ABC transporter in GenBank database release 90 (Altschul *et al.*, 1990, *J. Mol. Biol.*, 215: 403-410).

Degenerate PCR was performed using *Arabidopsis* genomic DNA as template. Amplification was for 45 cycles using the following thermal profile: 94°C

for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. A 0.6 kb PCR product was isolated, shown to hybridize strongly with a mixed probe encompassing the second NBF domain of *MRP1* and *YCF1*, and was cloned into pCRII vector (Invitrogen).

Sequence analysis verified that the deduced translation product of the *Arabidopsis* PCR product exhibited greatest similarity to *YCF1* and *MRP1* plus an unidentified 1.7 kb *Arabidopsis* EST (ATTS1246; Hofte *et al.*, 1993, *Plant J.*, 4: 1051-1061). In order to increase the likelihood of obtaining positive clones, a mixed probe consisting of the 0.6 kb PCR product and 1.6 kb EST was employed for further screens.

Eleven independent positive clones were obtained after screening approximately 3×10^5 plaques of a size-fractionated (3-6 kb) *Arabidopsis* cDNA library constructed in λ ZAPII (Kieber *et al.*, 1993, *Cell*, 72: 427-441) with the mixed probe. Restriction mapping confirmed that all 11 isolates corresponded to the same gene. The longest of these inserts, a 3.5 kb insert, designated *AtMRP2*, was subcloned and sequenced (Figure 13).

Since this isolate of *AtMRP2* was estimated to be missing approximately 1.5 kb of the 5' sequence of the ORF (assuming that the complete ORF of *AtMRP2* is similar in size to the ORFs of human *MRP1* and yeast *YCF1*), 500 bp of the most 5' sequence of *AtMRP2* was used to probe two *Arabidopsis* bacterial artificial chromosome (BAC) libraries, UCD and TAMU (Choi *et al.*, 1995, *Weeds World*, 2: 17-20) to isolate clones containing the missing sequence. This procedure yielded 8 BAC clones: U1L22, U8C12, U12A2, U23J22, U419, T9C22, T1B17 and T4K22. After digestion with *HindIII*, those fragments that hybridized with the 3.5 kb cDNA insert were introduced into pBluescript SK⁺ and sequenced. Two of these BAC clones (T1B17, T4K22) comprise a second *MRP1* plant homolog, designated *AtMRP1*, while the remainder (U1L22, U8C12, U12A2, U23J22, U419, T9C22) comprise *AtMRP2* (see below).

After establishing that an approximately 10 kb *HindIII* fragment from BAC clone U1L22 encompassed sequences identical to *AtMRP2*, a *BglIII* restriction fragment comprising the first 3 kb of the BAC clone was used to rescreen

approximately 2×10^6 plaques from the *Arabidopsis* λ ZAPII cDNA library. Twenty six independent positive clones were obtained and the one containing the longest cDNA insert, 5.2 kb, was sequenced.

5 Sequence analysis demonstrated that the 5.2 kb cDNA was not identical to *AtMRP2* but instead a very closely related gene. Designated *AtMRP1* (Figure 16), the 5.2 kb cDNA was 84.3% and 88.2% identical to *AtMRP2* at the nucleotide and amino acid levels, respectively. Importantly, *AtMRP1* is a full-length cDNA.

10 Having determined the complete sequence of the *AtMRP1* cDNA, it was possible to identify the initiation codon of the *AtMRP2* genomic clone, design a specific 5'-UTR primer and amplify the remaining 5' end of *AtMRP2* to generate a full-length cDNA. Thus, full-length cDNAs encoding *AtMRP2* and *AtMRP1* (Figures 13 and 16, respectively) and genomic clones corresponding to *AtMRP2* and *AtMRP1* have been generated (Figure 14 and 17, respectively). The deduced amino acid sequences of *AtMRP2* and *AtMRP1* are presented in Figures 15 and 18, respectively.

15 Expression of *AtMRP1* in *Saccharomyces cerevisiae*

The experiments described below establish that *AtMRP1* mediates the MgATP-dependent transport of GS-conjugates. The results of similar experiments on *AtMRP2* demonstrate that this gene product has the same transport capability.

20 The data presented herein establishes that *YCF1* from *Saccharomyces cerevisiae* encodes a 1,515 amino acid ATP-binding cassette (ABC) transporter protein which localizes to the vacuolar membrane and catalyzes MgATP-dependent GS-conjugate transport. Membrane vesicles from wild type (DTY165) cells contain two pathways for transport of the model GS-conjugate, DNP-GS: an MgATP-dependent, uncoupler-insensitive pathway and an electrically driven pathway. Membranes from
25 the mutant strains DTY167 and DTY168, harboring a deletion of the *YCF1* gene, are by contrast more than 90% impaired in MgATP-dependent, uncoupler-insensitive DNP-GS transport. Yeast strains lacking a functional *YCF1* gene therefore represent a model system for probing the GS-conjugate transport function of plant *YCF1/MRP1* homologs.

To test the transporter capacity of *AtMRP1* (the first clone for which a full-length cDNA was obtained) for conferring GS-conjugate transport, yeast strain DTY168 (disrupted for the *YCF1* gene) was transformed with an expression vector engineered to contain the coding sequence of *AtMRP1*. After selection of the transformants, membranes were prepared and assayed for MgATP-dependent, uncoupler-insensitive DNP-GS transport as described herein. The results establish that *AtMRP1* catalyzes GS-conjugate transport in a manner indistinguishable from the vacuolar GS-X pump.

Construction of the expression vector

In order to constitutively express the *AtMRP1* gene in *S. cerevisiae*, a derivative of the yeast-*E. coli* shuttle vector, pYES2 (Invitrogen), was constructed. Essentially, the 831 bp *XbaI/NotI* fragment encompassing the 3-phosphoglycerate kinase (*PGK*) promoter of plasmid pFL61 (Minet et al., 1992, Plant J. 2:417-422) was inserted between the *SpeI/NotI* restriction sites of pYES2. In so doing, the galactose-inducible yeast *GAL1* promoter of pYES2 was replaced by the constitutive yeast *PGK* promoter, *pPGK*. This plasmid, designated pYES3, is otherwise identical to pYES2. The gene to be expressed is inserted into the multiple cloning site located between the *PGK* promoter and *CYC1* termination sequences.

Preliminary experiments had established that the 5' untranslated region (UTR) of the original *AtMRP1* cDNA isolate diminished expression of the open reading frame in yeast. Thus, to maximize expression, the 127 bp 5'-UTR of *AtMRP1* was removed. For this purpose, pBluescript SK⁻-*AtMRP1* was digested with *HpaII/SnaBI* to delete 3045 bp of the internal sequence. The remaining 5 kb fragment from this digest was gel-purified and self-ligated to generate truncated *AtMRP1* cDNA as a template for PCR. One hundred pmol of *AtMRP1*-Nco primer (5'-AAACCGGTGCGGCCCGCCATGGGGTTTGAGCCGT-3') (SEQ ID NO:19) and 100 pmol of T3 primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO:20) were used to amplify a 2002 bp fragment of *AtMRP1* using Pfu DNA polymerase

(Stratagene). Amplification was for 30 cycles using the following thermal profile: 94°C for 15 seconds; 50°C for 15 seconds; and 72°C for 3.5 minutes.

The PCR product was gel-purified, digested with *SpeI* and cloned into the *EcoRV/SpeI* sites of pBluescript SK⁻ to generate pSK⁻ *AtMRP1*-Nco2. The 1227 bp *SphI/SpeI* fragment of this construct was then exchanged with the 4363 bp *SphI/SpeI* fragment of pBluescript pSK⁻ *AtMRP1* to generate pSK⁻ *AtMRP1*-Nco5. pYES-*AtMRP1*, lacking the 5' UTR, was constructed by digesting pSK⁻ *AtMRP1*-Nco5 with *XhoI/SpeI* to obtain a 5049 bp truncated *AtMRP1* gene fragment which was cloned into the *XhoI/XbaI* sites of pYES3. One kb of the 5' sequence of the *AtMRP1* insert of pYES3-*AtMRP1* was analyzed and was found to match exactly the sequence of the original cDNA clone.

Transformation of Yeast

S. cerevisiae strain DTY168 (MAT α *his6*, *leu2-3*, -112, *ura3-52* *ycf1::hisG*) was transformed with pYES3-*AtMRP1* or empty vector lacking the *AtMRP1* insert (pYES3) by the LiOAc/PEG method (Giest et al., 1991, Yeast 7:253-263) and selected for uracil prototrophy by plating on AHC medium containing tryptophan (Kim et al., 1994, *supra*).

Isolation of membrane vesicles

For the preparation of membrane vesicles, 15 ml volumes of stationary phase cultures of the transformants were diluted into 1 L of fresh AHC medium and grown to an OD_{600 nm} of about 1.2. Membrane vesicles were purified as described herein and in Kim et al. (1995, *supra*).

Measurement of DNP-GS uptake

DNP-GS uptake was measured as described herein in 200 μ l reaction volumes containing 3 mM ATP, 3 mM MgSO₄, 5 μ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of [³H]DNP-GS (17.4 mCi/mmol). Gramicidin-D (uncoupler) was included to abolish the H⁺-

electrochemical potential difference that would otherwise be established by the V-ATPase in media containing MgATP.

The results of this study

Membrane vesicles purified from pYES3-*AtMRP1*-transformed DTY168 cells exhibit an approximately 4-fold increase in MgATP-dependent, uncoupler-insensitive [^3H]DNP-GS uptake by comparison with membrane vesicles purified from DTY168 cells transformed with empty vector (Figure 19). When measured at a DNP-GS concentration of 61.3 μM , the initial rates of uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed cells were 0.4 nmol/mg/minute and 0.1 nmol/mg/minute, respectively (Figure 19).

The concentration dependence and vanadate inhibitory of uptake verify direct participation of *AtMRP1*. MgATP-dependent, uncoupler-insensitive uptake by membrane vesicles purified from the pYES3-*AtMRP1* transformants increases as a single hyperbolic function of DNP-GS concentration to yield K_m and V_{max} values of $48.7 \pm 15.4 \mu\text{M}$ and $6.0 \pm 1.7 \text{ nmol/mg/10 minutes}$, respectively (Figure 19). pYES3-*AtMRP1*-dependent DNP-GS uptake decreases as a single exponential function of the concentration of the phosphoryl transition state analog vanadate, to yield an I_{50} of $8.3 \pm 3.3 \mu\text{M}$ (Figure 20). By contrast, the apparent K_m for DNP-GS uptake by membrane vesicles purified from pYES3-transformed DTY168 cells is in excess of 500 μM and uptake is insensitive to vanadate.

On the basis of its sequence characteristics and the results of these experiments, *AtMRP1* encodes the vacuolar GS-X pump. The increases in uptake following the introduction of plasmid borne *AtMRP1* into yeast (ca. 4 nmol/mg/20 minutes) are commensurate with the rates of MgATP-dependent, uncoupler-insensitive DNP-GS uptake measured in vacuolar membrane vesicles purified from plant sources (2.3, 3.8, 18.2, 5.8, and 2.1 nmol/mg/20 minutes for *Arabidopsis* leaf, *Arabidopsis* root, *Beta vulgaris* storage root, *Vigna radiata* hypocotyl and *Zea mays* root, respectively) (Table III in Li *et al.*, 1995, *supra*). The K_m for DNP-GS transport by heterologously

expressed AtMRP1 is similar to that reported for the endogenous GS-X pump of plant vacuolar membranes ($81.3 \pm 41.8 \mu\text{M}$, Li *et al.*, 1995, *supra*). The I_{50} for inhibition of AtMRP1-dependent DNP-GS transport by vanadate coincides with the I_{50} for inhibition of the endogenous vacuolar GS-X pump of plant cells ($7.5 \pm 3.9 \mu\text{M}$, Li *et al.*, 1995, *supra*).

Having confirmed that the endogenous vacuolar GS-X pump of *S. cerevisiae* is lacking in the *ycf1* Δ mutant strains, DTY168 and DTY167 (Li *et al.*, 1995, *supra*), and in any case has a markedly lower K_m for DNP-GS and is 6 to 8-fold less sensitive to vanadate than the plant cognate, these findings establish that AtMRP1 *per se* is responsible for the MgATP-dependent, uncoupler-insensitive transport measured in these experiments. Given that heterologous expression of *AtMRP1* alone is sufficient for DNP-GS transport in DTY168 cells, it is concluded that one of the GS-X pumps of *Arabidopsis* has been cloned in its entirety.

Sequence comparisons of MRP1, cMOAT, YCF1, AtMRP1 and AtMRP2 with other members of the ABC transporter superfamily reveal two major subgroups. One group contains MRP1, cMOAT, YCF1, AtMRP1, AtMRP2 and the *Leishmania* P-glycoprotein-related molecule (Lei/PgpA). The other group contains the MDRs, the major histocompatibility complex transporters and STE6. However, of all the ABC transporters defined to date, cMOAT, YCF1, AtMRP1 and AtMRP2 exhibit the closest resemblance to each other. Unlike the similarities between the GS-X pump subgroup, Lei/PgpA and CFTR, which center on the nucleotide binding folds (NBFs), the similarities between the GS-X pump members cMOAT, YCF1, AtMRP1 and AtMRP2 are found throughout the sequence. GS-X family members are 40-45% identical (60-65% similar) at the amino acid level, possess NBFs with an equivalent spacing of conserved residues and are colinear with respect to the location, extent and alteration of putative transmembrane spans and extramembrane domains. Two features of members of the GS-X pump family that distinguish them from other ABC transporters are their possession of a central truncated CFTR-like regulatory domain

rich in charged amino acid residues and an approximately 200 amino acid residue N-terminal extension.

A hydropathy alignment of AtMRP1, AtMRP2, YCF1, HmMRP1, and RiCMOAT is shown in Figure 21. Note the following: (i) The almost exact equivalence of AtMRP1 and AtMRP2 with respect to the alternation of hydrophobic and hydrophilic stretches. (ii) The close correspondence of AtMRP1 and AtMRP2 with all of the other members of the MRP1/YCF1/cMOAT subclass of ABC transporters in terms of the overall hydropathy profiles. (iii) The "signature" profile for the N-terminal 200 amino acid residues of all of the sequences shown, which is unique to the MRP1/YCF1/cMOAT subclass. Hydropathy was computed according to Kyte and Doolittle (1982, *J. Mol. Biol.* 46:105-132) over a running window of 15 amino acid residues. Hydrophobic stretches of sequence fall below the line and hydrophilic stretches fall above the line.

In Figure 22 there is depicted domain comparisons between AtMRP1, ScYCF1, HmMRP1, RiCMOAT, RbEBCR and HmCFTR. The domains indicated are the N-terminal extension (NH₂), first and second transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH). This comparison is also tabulated in Tables 8 and 9.

TABLE 8: Identity and similarity analysis of putative domains of AtMRP1 against AtMRP2 ScYCF1, HmMRP1, RiCMOAT, HmCFTR and RbEBCR, ScYCF1, *Saccharomyces cerevisiae* YCF1; HmMRP1, human MRP1; RiCMOAT, rat cMOAT; HmCFTR, human CFTR; RbEBCR, rabbit EBCR. The domains identified are N-terminal extension (NH₂), transmembrane segments 1 and 2 (TM1 and TM2, respectively), CFTR-like regulatory domain (R), nucleotide binding folds 1 and 2 (NBF1 and NBF2, respectively) and C-terminus (COOH). Similarity was calculated as described herein over the sequence segments indicated in Table 9.

SEQUENCE	DOMAIN	OVERALL	NH ₂	TM1	NBF1
AtMRP2	Identity	87.0	74.4	90.4	92.1
	Similarity	93.7	85.2	96.1	96.7
ScYCF1	Identity	36.1	13.3	32.2	50.0

SEQUENCE	DOMAIN	OVERALL	NH ₂	TM1	NBFI
	<i>Similarity</i>	55.4	32.9	52.6	75.0
HmMRP1	<i>Identity</i>	41.5	16.2	37.4	58.0
	<i>Similarity</i>	63.3	34.8	57.5	78.7
RtCMOAT	<i>Identity</i>	38.6	19.6	33.8	58.7
	<i>Similarity</i>	60.2	36.7	61.0	80.0
HmCFTR	<i>Identity</i>	29.2	0	22.8	40.7
	<i>Similarity</i>	55.1	0	47.8	62.0
RbEBCR	<i>Identity</i>	38.9	17.2	34.5	62.4
	<i>Similarity</i>	60.4	34.9	61.6	82.6
SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP2	<i>Identity</i>	80.5	86.9	91.3	89.4
	<i>Similarity</i>	89.8	94.2	96.5	94.4
ScYCF1	<i>Identity</i>	33.9	34.7	34.7	58.1
	<i>Similarity</i>	59.5	57.9	57.9	71.8
HmMRP1	<i>Identity</i>	31.6	31.9	61.9	48.3
	<i>Similarity</i>	50.4	56.3	72.8	69.0
RtCMOAT	<i>Identity</i>	33.9	34.4	60.7	50.0
	<i>Similarity</i>	50.0	58.8	75.7	67.2
HmCFTR	<i>Identity</i>	45.7	22.3	39.5	28.1
	<i>Similarity</i>	75.0	51.3	61.6	58.4
RbEBCR	<i>Identity</i>	35.6	34.1	61.9	43.0
	<i>Similarity</i>	51.7	59.1	74.0	62.7

TABLE 9. Positions and sizes of segments of sequence analyzed in Table 8.

SEQUENCE	DOMAIN	OVERALL	NH ₂	TM1	NBF1
AtMRP1	<i>Position</i>		1-223	224-631	634-782
	<i>Size</i>	1622	223	407	148
AtMRP2	<i>Position</i>		1-223	224-631	634-782

SEQUENCE	DOMAIN	OVERALL	NH ₂	TM1	NBF1
	Size	1622	223	407	148
ScYCF1	Position		1-210	211-645	646-787
	Size	1515	210	435	142
HmMRP1	Position		1-240	241-660	661-810
	Size	1531	240	420	150
RtCMOAT	Position		1-192	193-648	649-799
	Size	1540	192	456	151
HmCFTR	Position		0	1-440	441-590
	Size	1481	0	440	150
RbEBCR	Position		1-193	194-651	652-800
	Size	1562	193	458	149
SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP1	Position	783-900	901-1244	1245-1417	1418-1622
	Size	117	343	172	205
AtMRP2	Position	783-905	906-1249	1250-1422	1423-1622
	Size	122	343	172	200
ScYCF1	Position	788-936	937-1279	1280-1453	1454-1515
	Size	149	343	174	163
HmMRP1	Position	811-960	961-1300	1301-1473	1474-1531
	Size	150	340	173	59
RtcMOAT	Position	800-960	961-1302	1303-1476	1477-1541
	Size	161	342	174	65
HmCFTR	Position	591-847	848-1217	1218-1389	1390-1481
	Size	256	371	172	92
RbEBCR	Position	801-961	962-1304	1305-1477	1478-1562
	Size	161	343	173	86

As is apparent from the data presented above, there is significant homology between similar domains among AtMRP-related proteins. In particular, the N-terminal and R domains share significant homology among the AtMRP-related proteins tested. These data establish that in addition to primary sequence, the secondary structure of the molecule plays a significant role in GS-X pump function.

It should be appreciated that *AtMRP1* and *AtMRP2* constitute a family of genes in *Arabidopsis*, wherein various members of the family have different substrate specificities as demonstrated by the next set of experiments.

Substrate Preferences of AtMRP1 and AtMRP2

To examine the substrate preferences of AtMRP1 and AtMRP2, the following experiments were performed.

Isolation of Bn-NCC-1

[¹⁴C]Bn-NCC-1 (33.3 mCi/mmol) was extracted from senescent cotyledons of rape (*Brassica napus*) and was purified by preparative HPLC (Krautler *et al.*, 1992, *Plant Physiol. Biochem.* 30:333-346). Determination of the purity of the final preparation by analytical HPLC and enumeration of concentration and specific radioactivity (33.3 mCi/mmol) were performed according to Hinder *et al.* (1966, *J. Biol. Chem.* 271:27233-27236). Unlabeled Bn-NCC-1 was isolated from fully senescent cotyledons of excised shoots that had been maintained in complete darkness for 1 week.

Measurement of transport

Cells were grown and vacuolar membrane-enriched vesicles were prepared as described (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635-). Uptake of [¹⁴C]Bn-NCC-1, [³H]C3G-GS, [³H]DNP-GS, [³H]GSSG, [¹⁴C]metolachlor or [³H]taurocholate was measured routinely in 200 μ l reaction volumes containing membrane vesicles (10-20 μ g protein), 3 mM ATP, 3 mM MgSO₄, 5 μ M gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine phosphate kinase, 50 mM KCl, 1mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated

concentrations of transport substrate. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol/3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate filters (pore size 0.45 μ m). The filters were rinsed twice with wash medium, and the retained radioactivity was determined by liquid scintillation counting. Nonenergized uptake was estimated by the same procedure except that ATP was omitted from the uptake medium.

The effect of taurocholate on the release of [3 H]DNP-GS from membrane vesicles that had been allowed to mediate AtMRP2-dependent accumulation of this compound during a preceding uptake period was determined. This was accomplished by rapid depletion of ATP from the uptake medium using a hexokinase trap (glucose + ATP \rightarrow glucose-6-phosphate + ADP) and measurements of the decrease in vesicular radiolabel in the presence or absence of taurocholate. Membranes from DTY168/pYES3-AtMRP2 cells were incubated for 10 minutes in standard uptake medium containing 61.3 μ M [3 H]DNP-GS after which time 200 mM glucose and 50 units/ml hexokinase (Type F-300 from baker's yeast) were added. After incubation for a further 2 minutes, taurocholate (50 μ M) or Triton X-100 (9.01% v/v) was added and release of vesicular [3 H]DNP-GS was measured as described. Control samples were treated identically except that no additions were made after the initial 10 minute incubation period.

Substrate Preferences

The absence of AtMRP2-dependent transport from DTY168 and DTY168/pYES3 membranes and the selective inhibition of this system by micromolar concentrations of vanadate, established that AtMRP2-dependent transport may be measured in two ways. This may be accomplished by assessing the difference between the rates of MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP2 membranes by comparison with DTY168 or DTY168/pYES3 membranes, or by assessing the vanadate-sensitive component of MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP2 membranes. Because the results were

qualitatively and quantitatively similar whichever method was used, "AtMRP2-dependent" transport as used in this section refers to uptake which is measured as the increment consequent on transformation of DTY168 cells with pYES3-AtMRP2 *versus* pYES3.

5 Application of this methodology to vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2- *versus* pYES3-transformed DTY168 cells and expansion of the transport assays to measurements of the concentration dependence of [3H]DNP-GS, [3H]GSSG, [14C]metolachlor-GS, [14C]Bn-NCC-1 and [3H]taurocholate uptake, demonstrated that the substrate preferences and maximal transport capacities of 10 AtMRP2 and AtMRP1 differed markedly. While uptake of all of the GSH derivatives examined conformed to Michaelis-Menten kinetics, the V_{max} values for AtMRP2-dependent uptake were consistently severalfold greater than those for AtMRP1-dependent uptake. The V_{max} values for AtMRP2-dependent uptake of [3H]DNP-GS, [3H]GSSG and [14C]metolachlor-GS were 16.3 ± 3.1 , 38.1 ± 3.2 and 136.0 ± 28.1 15 nmol/mg/10 min, respectively; the corresponding values for AtMRP1 were 8.2 ± 1.6 , 6.8 ± 1.1 and 17.5 ± 5.2 nmol/mg/10 min. With the exception of [3H]GSSG whose K_m for AtMRP1-dependent uptake ($21.9.2 \pm 58.3 \mu M$) was three times greater than that for AtMRP2-dependent uptake ($73.0 \pm 15.1 \mu M$), the K_m values estimated for AtMRP2 and AtMRP1 were very similar (65.7 ± 29.8 *versus* $63.6 \pm 36.5 \mu M$ for metolachlor-GS).

20 Single concentration ($50 \mu M$) measurements of uptake of the glutathionated anthocyanin, cyanin-3-glucoside-GS (C3G-GS), demonstrated an approximately 6-fold greater capacity of AtMRP2 for transport of this compound (rate = 48.4 ± 2.2 nmol/mg/10 min) by comparison with AtMRP1 (rate = 7.9 ± 0.7 nmol/mg/10min).

25 In no case was MgATP-dependent, uncoupler-insensitive uptake of the unconjugated precursors of the GS-compounds, DNP, GSH, metolachlor and C3G detectable.

Neither AtMRP2 nor AtMRP1 catalyzed the uptake of [3H]taurocholate. Transformation of DTY168 cells with either pYES3-AtMRP2 or pYES3-AtMRP1

conferred little or no increase in the capacity of vacuolar membrane-enriched vesicles for [^3H]taurocholate uptake over that measured with vesicles prepared from pYES3-transformed cells. The results of these experiments are presented in Table 10.

Table 10. Kinetic parameters for uncoupler-insensitive AtMRP1- and AtMRP2-dependent transport of GS-derivatives, *Bn*-NCC-1 and taurocholate.

Compound	AtMRP1		AtMRP2	
	K_m	V_{max}	K_m	V_{max}
DNP-GS	73.8 ± 18.8	8.2 ± 1.6	65.7 ± 29.8	16.3 ± 3.1
GSSG	219.2 ± 58.3	6.8 ± 1.1	73.0 ± 15.1	38.1 ± 3.2
Metolachlor-GS	63.6 ± 36.5	17.5 ± 5.2	75.1 ± 31.6	136.0 ± 28.1
<i>Bn</i> -NCC-1	-----Linear-----		15.2 ± 2.3	63.1 ± 2.5
Taurocholate	-----Linear-----		-----Linear-----	

MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP1, DTY168/pYES3-AtMRP2 and DTY168/pYES3 membranes was measured as described herein. The K_m and V_{max} values were estimated by fitting the data to a single Michaelis-Menten function by nonlinear least squares analysis. Values shown are means \pm SE.

The 2- to 8-fold greater capacity of AtMRP2 *versus* AtMRP1 for transport of the compounds examined was not attributable to differences in the levels of expression of their cDNAs from the *PGK* gene promoter of pYES3. Quantitative RT-PCR of equivalent amounts of total RNA extracted from DTY168/pYES3-AtMRP2 and DTY168/pYES3-AtMRP1 cells yielded similar levels of the 800 bp PCR

amplification product predicted from the sequences of the oligonucleotide primers used. Since neither amplification product was generated when PCR was performed without reverse transcription or when total RNA from DTY168/pYES3 cells was employed as template, contamination by genomic DNA and/or RT-PCR of transcripts other than those from *AtMRP2* or *AtMRP1*, respectively, was not responsible for the observed results.

Anomalous interactions between candidate transport substrates

Two critical properties of *AtMRP2* were its capacity for the simultaneous transport of GS-conjugates and *Bn*-NCC-1 and its pronounced sensitivity to inhibition by taurocholate. Simultaneous measurements of [¹⁴C]*Bn*-NCC-1 and [³H]DNP-GS uptake by membrane vesicles purified from DTY168/pYES3-*AtMRP2* cells revealed parallel accumulation of both compounds with little or no interference of the transport of one by the other. *AtMRP2*-dependent uptake of [¹⁴C]*Bn*-NCC-1 at an extravesicular concentration equivalent to its K_m value (15 μ M,) was nearly three times less sensitive to DNP-GS than would be predicted if this GS-conjugate were a competitor. If DNP-GS were a simple competitive inhibitor such that its K_m value (66 μ M) approximated its K_i value for the inhibition of *Bn*-NCC-1 uptake, 120 μ M DNP-GS would be expected to inhibit [¹⁴C]*Bn*-NCC-1 uptake by 48% but this was not observed. DNP-GS concentrations in excess of 120 μ M decreased [¹⁴C]*Bn*-NCC-1 uptake by less than 18%. Reciprocally, the concentration-dependence of *AtMRP2*-mediated [³H]DNP-GS uptake was not affected appreciably by *Bn*-NCC-1. The K_m and V_{max} values for *AtMRP2*-dependent [³H]DNP-GS uptake in the presence of 15 μ M *Bn*-NCC-1 ($80.5 \pm 28.6 \mu$ M and 18.3 ± 1.6 nmol/mg/10/min) were similar to those measured in its absence.

Although neither *AtMRP2* nor *AtMRP1* transported taurocholate, *AtMRP2*-mediated transport was selectively inhibited by this compound. *AtMRP1*-dependent [³H]DNP-GS uptake was relatively insensitive to taurocholate ($I_{50} > 250 \mu$ M) but the uptake of both [³H]DNP-GS and [¹⁴C]*Bn*-NCC-1 by *AtMRP2* was strongly inhibited ($I_{50(\text{DNP-GS uptake})} = 27 \pm 1.3 \mu$ M; $I_{50(\text{Bn-NCC-1 uptake})} = 49.5 \pm 0.3 \mu$ M).

Taurocholate at the concentrations employed appeared to exert its effect on AtMRP2-mediated transport by inhibiting pump activity directly rather than by increasing background membrane permeability and decreasing net influx by increasing passive DNP-GS efflux. Addition of taurocholate at a concentration (50 μ M) sufficient to inhibit AtMRP2-dependent [3 H]DNP-GS uptake by 70% to DTY168/pYES3-AtMRP2 vesicles that had accumulated [3 H]DNP-GS for 10 minutes before arresting pump action by ATP depletion using a hexokinase trap, did not accelerate the efflux of intravesicular 3 H-label over that measured on vesicles subject to a hexokinase trap in the absence of taurocholate. Imposition of a hexokinase trap and addition of a concentration of detergent (Triton X-100; 0.01% v/v) known to permeate these membranes (Zhen *et al.*, 1997, *J. Biol. Chem.* 272:22340-22348), on the other hand, increased the rate and extent of release of the [3 H]DNP-GS accumulated during the preceding 10 minute uptake period by more than 3-fold *versus* DTY168/pYES3-AtMRP2 vesicles treated with hexokinase alone or hexokinase plus taurocholate.

The high capacity of AtMRP2 for the transport of large amphipathic anions other than GS-conjugates (i.e., *Bn*-NCC demonstrates that one pump can assume more than one of the several ABC transporter-like functions identified in plants to date. In the case of AtMRP2, this includes transport activity directed to a broad-range GS-conjugate pump and a chlorophyll metabolite pump. Thus, on the one hand, the high capacity of heterologously expressed AtMRP2, and to a lesser extent AtMRP1, for the transport of metolachlor-GS, and by extension GS-conjugates of other herbicides to glutathionation, is consistent with the molecular identification of transporters capable of removing these and related compounds from the cytosol. On the other hand, the high capacity of AtMRP2 for the transport of *Bn*-NCC is consistent with the identification of an element capable of contributing to the further metabolism and eventual removal of tetrapyrrole derivatives generated during leaf senescence from the cytosol.

Vacuolar uptake of glutathionated medicarpin by the glutathione conjugate pump

A key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. Accordingly, most studies of plant-pathogen interactions in the Leguminosae have centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, it is essential that they are accumulated in the plant in a site which is sequestered (*i.e.*, isolated) from the cytoplasm.

The following experiments describe uptake of free [^3H]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung bean (*Vigna radiata*). This uptake is slow and relatively insensitive to MgATP. However, after incubation with glutathione and a total glutathione-S-transferase preparation from maize (*Zea mays*), [^3H]medicarpin uptake occurs at a rate which is 8-fold faster in the presence, as opposed to the absence of MgATP. MgATP-dependent uptake of glutathione/glutathione-S-transferase pretreated [^3H]medicarpin is only slightly inhibited by uncoupler (gramicidin D), but is strongly inhibited by vanadate and the model glutathione-S-conjugate, S-(2,4-dinitrophenyl)glutathione. These results demonstrate that the MgATP-energized glutathione-conjugate pump identified herein in the membrane preparation is capable of high affinity, high capacity transport of glutathionated isoflavonoid phytoalexins. The experimental procedures and results of these experiments are now described.

Preparation of [^3H]medicarpin

[^3H]medicarpin was produced by base-catalyzed tritium exchange from $^3\text{H}_2\text{O}$ using unlabeled medicarpin isolated from fenugreek (*Trigonella foenumgraecum*) seedlings exposed to 3 mM CuCl_2 .

GST purification and conjugation of medicarpin

Two-week old maize (*Zea mays*) B73N seedlings were grown under continuous light at 21 °C. Twenty four hours prior to harvesting, the seedlings were exposed to a mild treatment with 2,4-dichlorophenoxyacetic acid and atrazine to stimulate GST expression (Timmerman, 1989, *Physiol. Plant* 77:465). Two-gram samples of root and shoot tissue were ground to homogeneity in 50 ml of 500 mM sodium phosphate buffer, pH 7.8 (Buffer A). The extract was centrifuged at 7,000 x g for 10 minutes at 4 °C and the resulting supernatant was filtered through Miracloth and mixed with 2 ml of S-hexylglutathione-conjugated agarose beads (Sigma). After incubation for 5 minutes at 21 °C, the beads were sedimented by centrifugation at 500 x g at 4 °C. The supernatant was discarded and the beads were resuspended in 2.5 ml of prechilled Buffer A and centrifuged again. Bound GST was eluted by resuspension of the beads in 2 ml Buffer B (20 mM GSH, 500 mM sodium phosphate, pH 7.8) and incubation for 5 minutes at 21 °C. The beads were sedimented by centrifugation at 500 x g and the supernatant was assayed for GST activity (Mannervick *et al.*, 1981, *Methods Enzymol.*, 77:231).

[³H]medicarpin (0.5 µCi, 4.5 Ci/mol) was conjugated with GSH by incubation with 25 µl of total purified maize GSTs for 3 hours at 21 °C in the dark. Control, unconjugated samples were prepared by mixing [³H]medicarpin (0.5 µCi) with cold Buffer B and immediately freezing the mixture in liquid nitrogen.

Synthesis of S-(2,4-dinitrophenyl)glutathione (DNP-GS)

DNP-GS was synthesized from 1-chloro-2,4-dinitrobenzene and GSH by a modification of the enzymatic procedure of Kunst *et al.*, (1989, *Biochim. Biophys. Acta* 983:123; Li *et al.*, 1995, *supra*).

Preparation of vacuolar membrane vesicles

Vacuolar membrane vesicles were purified from etiolated hypocotyls of *V. radiata* cv. Berken as described (Li *et al.*, *Plant Physiol.* 109: 1257, Li *et al.*, 1995, *supra*).

Measurement of uptake

Unless otherwise indicated, [^3H]medicarpin or [^3H]medicarpin-GS uptake was measured at 25°C in 200 μl reaction volumes containing 3 mM ATP, 3 mM MgSO_4 , 10 mM creatine phosphate, 16 U/ml creatine kinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol and 25 mM Tris-Mes buffer, pH 8.0. Uptake was initiated by the addition of 12 μl membrane vesicles (30-40 μg protein) and brief mixing of the samples on a vortex mixer. Uptake was terminated by the addition of 1 ml ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted HA cellulose nitrate filters (pore diameter 0.45 μm). The filters were rinsed twice with a 1 ml ice-cold wash medium, air-dried and radioactivity was determined by liquid scintillation counting.

Protein estimations and source of commercial chemicals was as described herein.

Results

Appreciable MgATP-dependent uptake of [^3H]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung was dependent on preincubation of this compound with GSH and GSTs. Free [^3H]medicarpin incubated in the presence of GSH in the absence of GSTs was taken up at 16.7 ± 3.6 and 7.4 ± 1.3 nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25). In contrast, [^3H]medicarpin-GS synthesized by incubating [^3H]medicarpin with GSH in the presence of affinity-purified maize GSTs, was taken up at 81.0 ± 13.3 and 11.3 ± 0.4 nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25).

MgATP-dependent [^3H]medicarpin-GS uptake was strongly inhibited by vanadate and DNP-GS but was relatively insensitive to uncouplers. Whereas inclusion of vanadate (10 μM) or DNP-GS (100 μM) in the assay medium inhibited [^3H]medicarpin uptake by more than 85%, addition of the ionophore, gramicidin D, diminished uptake by only 17% (Table 11).

Table 11. Effects of different inhibitors on [3 H]medicarpin-GS uptake by vacuolar membrane vesicles. [3 H]medicarpin-GS was added at a concentration of 65 μ M. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Gramicidin-D, vanadate and DNP-GS were added at concentrations of 5 μ M, 10 μ M and 100 μ M, respectively. Values outside parentheses are means \pm SE ($n = 3$); values inside parentheses are rates of uptake expressed as percentage of control.

TREATMENT	[3 H]Medicarpin-GS Uptake (nmol/mg/10 minutes)	
	+MgATP	-MgATP
Control	85.6 \pm 13.3 (100)	16.7 \pm 6.0 (100)
+ Gramicidin-D	71.2 \pm 3.0 (83.2)	13.2 \pm 2.1 (79.0)
+ Gramicidin-D + vanadate	12.9 \pm 0.9 (15.1)	17.4 \pm 0.5 (104.2)
+ Gramicidin-D + DNP-GS	11.7 \pm 2.8 (13.7)	5.6 \pm 3.1 (33.5)

MgATP-dependent, uncoupler-insensitive uptake increases as a single Michaelian function of [3 H]medicarpin-GS concentration to yield K_m and V_{max} values of 21.5 \pm 15.5 μ M and 77.8 \pm 23.3 nmol/mg/20 minutes, respectively (Figure 26).

Direct involvement of the GS-X pump in the accumulation of [3 H]medicarpin-GS by vacuolar membrane vesicles is therefore evident at three levels: (i) Glutathionation of medicarpin selectively increases MgATP-dependent uptake. MgATP-independent uptake is marginally affected by glutathionation but MgATP-dependent uptake is stimulated by approximately six-fold confirming that medicarpin-GS is the transported species and MgATP is the energy source. (ii) Uptake is directly energized by MgATP. The inability of uncoupler to markedly inhibit [3 H]medicarpin-GS uptake implies that the H^+ -electrochemical gradient that would otherwise be established by the vacuolar H^+ -ATPase in the presence of MgATP does not drive uptake. Rather, the pronounced inhibition of MgATP-dependent uptake exerted by vanadate agrees with the notion that GS-X-mediated uptake is strictly dependent on ATP hydrolysis and formation of a phosphoenzyme intermediate (Martinoia *et al.* 1993, *supra*; Li *et al.*, 1995, *supra*), (iii) [3 H]medicarpin-GS and the model GS-X pump substrate DNP-GS, whose transport has been exhaustively analyzed in this system as

described herein, compete for uptake indicating that both are transported by the same moiety.

The efficacy of medicarpin-GS as a substrate for the vacuolar GS-X pump is striking. Even though the K_m for medicarpin-GS uptake is undoubtedly an overestimate, since the yield from the conjugation reaction was not enumerated, it is nevertheless 2 to 25-fold lower than those estimated previously for DNP-GS, C3G-GS (80 and 46 μ M in this system), glutathione-S-N-ethylmaleimide (500 μ M) and metolachlor-GS (40-60 μ M, barley vacuoles; Martinoia *et al.*, 1993, *supra*). Moreover, the capacity of the GS-X pump for medicarpin-GS uptake is high ($V_{max} = 78$ nmol/mg/20 minutes) versus DNP-GS ($V_{max} = 12$ nmol/mg/20 minutes) and comparable to that estimated for C3G-GS ($V_{max} = 45$ nmol/mg/minute). Thus, while maize anthocyanin was the first natural substrate shown to be vacuolarly sequestered through the concerted actions of cytosolic GSTs and the vacuolar GS-X pump in plants (Marrs *et al.*, 1995, *Nature*, 375:397 and data contained herein), medicarpin, and presumably other isoflavonoid phytoalexins, is equally strong a candidate.

These data suggest that the GSTs which are induced following the hypersensitive response to avirulent fungal pathogens likely serve to facilitate the vacuolar storage of antimicrobial compounds in the healthy cells surrounding the lesion.

Transport of glutathionated anthocyanins and auxins by the vacuolar GS-X pump of plant cells

The data which are now described demonstrate that the vacuolar GS-X pumps of corn (*Zea mays*) roots and etiolated hypocotyls of mung bean (*Vigna radiata*) transport the anthocyanin cyanidin-3-glucoside (C3G), and the phytohormone, indole-3-acetic acid (IAA), after conjugation with glutathione. Whereas the unconjugated forms of these compounds undergo negligible uptake into vacuolar membrane vesicles, both C3G-GS and IAA-GS are subject to high rates of MgATP-dependent, uncoupler-insensitive uptake (Figure 27 and Table 12). IAA-GS and C3G-GS uptake approximates Michaelis-Menten kinetics to yield K_m values in the micromolar range

and V_{\max} values 7- to 40-fold greater than those measured with the artificial transport substrate, DNP-GS (Table 12 and Li *et al.*, 1995, *supra*). Uptake of both conjugates is inhibited by DNP-GS and vanadate in a manner consistent with mediation by the GS-X pump (Figure 27 and Table 13). In contrast, glutathionated abscissic acid (ABA-GS) is a poor substrate for the GS-X pump: uptake is relatively slow and only saturates at high substrate concentrations (Figure 27 and Table 13).

Table 12. Summary of kinetic parameters for MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* and roots of *Z. mays*. Kinetic parameters (K_m , μM ; V_{\max} , $\text{nmol/mg}/10 \text{ min}$) were computed from the data shown in Figure 27 by nonlinear least squares analysis. Values shown are means \pm SE.

PARAMETER	C3G-GS		IAA-GS
	<i>V. radiata</i>	<i>Z. mays</i>	<i>V. radiata</i>
K_m	45.7 \pm 14.0	39.5 \pm 16.6	36.0 \pm 16.7
V_{\max}	45.3 \pm 6.5	79.1 \pm 14.7	17.7 \pm 5.8
PARAMETER	IAA-GS	ABA-GS	
	<i>Z. mays</i>	<i>V. Radiata</i>	<i>Z. mays</i>
K_m	47.7 \pm 19.6	>1000	128.8 \pm 79.1
V_{\max}	30.0 \pm 4.9	22.9 \pm 9.2	4.0 \pm 1.4

It has been known for some time that the characteristic bronze coloration of *Bronze-2* (*bz2*) mutants is a consequence of the accumulation of cyanidin-3-glucoside in the cytosol. In wild type (*Bz2*) plants, anthocyanins are transported into the vacuole and become purple or red whereas in *bz2* plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a brown ("bronze") pigment. However, until the present invention, the exact molecular basis of this lesion was unknown. Since *Bz2* encodes a GST responsible for conjugating anthocyanin with GSH (Marrs *et al.*, 1995) and glutathionated anthocyanins are transported by the vacuolar GS-X pump, the

experiments described herein explain the bronze phenotype. Being defective in the glutathionation of anthocyanins, *bz2* mutants are unable to pump these pigments from the cytosol into the vacuole lumen; a conclusion borne out by the ability of the GS-X pump inhibitor, vanadate, to phenocopy the *bz2* lesion in wild type protoplasts and the efficacy of cyanidin-3-glucoside-GS as a substrate for the plant vacuolar GS-X pump *in vitro*, as the data presented herein establish.

The concept underlying the above-described experiments on phytohormones is that they may be subject to metabolic interconversions and compartmentation analogous to those deduced for anthocyanins. On the one hand, it is now established that C3G must be glutathionated before it can be transported into the vacuole. On the other hand, it is evident that most of the vacuolar anthocyanins of intact plants are not stored in this form. Instead, they are subject to long term storage as their malonyl derivatives. It is therefore apparent that while C3G-GS is a short-lived but necessary intermediate for vacuolar anthocyanin compartmentation, it is not the terminal product of this process. The experiments with auxins further illustrate this principle by demonstrating that IAA is susceptible to glutathionation and that the resultant IAA-GS conjugate is transported by the vacuolar GS-X pump in a MgATP-dependent, uncoupler-insensitive, vanadate-inhibitible manner. Thus, even though IAA-GS derivatives have not been detected *in planta*, this does not exclude the possibility that they are short-lived transport intermediates necessary for subsequent vacuolar processing of this class of compounds.

Table 13. Concentrations of DNP-GS and vanadate required to inhibit MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by 50% (I_{50} values) by vacuolar membrane vesicles purified from etiolated hypocotyls of <i>V. radiata</i> and roots of <i>Z. mays</i> . I_{50} values (μ M) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential. ND, not determined.			
	C3G-GS	IAA-GS	ABA-GS
COMPOUND	<i>V. radiata</i>	<i>Z. mays</i>	<i>V. radiata</i>
Vanadate	7.9	8.2	6.5
DNP-GS	103.5	112.4	124.2
	C3G-GS	IAA-GS	ABA-GS
COMPOUND	<i>Z. mays</i>	<i>V. radiata</i>	<i>Z. mays</i>
Vanadate	5.5	ND	>150
DNP-GS	109.8	ND	231.5

Generation of a Transgenic Plant Comprising a Transgene Encoding a GS-X Pump

To generate a transgenic plant comprising a gene encoding YCF1, the following experiments were performed. The binary vector pROK-YCF1, encoding wild type YCF1 was constructed. The sense orientation of the inserts with respect to the CaMV 35S promoter of pROK (Baulscombe *et al.*, 1986, *Nature* 321:446-449) was confirmed and these constructs, as well as empty vector (pROK) controls, were transformed into *Agrobacterium* strain C58 by electroporation (Ausubel *et al.*, 1992, *Current Protocols in Molecular Biology*, pp 27-28).

Kanamycin-resistant *Agrobacterium* transformants were isolated, the integrity of the constructs in the bacterial recipient was established by PCR and *Arabidopsis* roots were inoculated with the transformants (Huang *et al.*, 1992, *Plant Mol. Biol.* 10:372-384). The resulting rosette shoots generated on selective medium

were transferred to root-inducing medium for regeneration. Stable insertion of the sense strands and constitutive expression of *YCF1* and *YCF1::HA* was demonstrated in the kanamycin-resistant *Arabidopsis* transformants, by probing Southern blots with *YCF1* and pROK sequences and by Northern analyses, respectively.

5 An association between *YCF1* expression and altered xenobiotic resistance was tested by screening multiple T2 generation pROK-*YCF1*, pROK-*YCF1-HA* and pROK empty vector transformant lines for tolerance to cadmium salts and the GS-conjugable xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB). CDNB has three advantages for studies of this type: (i) It is an established plant toxin (Li *et al.*, 1995, *Plant Physiol.* 109:117-185); (ii) The kinetics of transport of its glutathionated derivative, DNP-GS, as well characterized for *YCF1* (Li *et al.*, 1996, *J. Biol. Chem.* 271:6509-6517) and the endogenous GS-X pump (Li *et al.*, 1995, *Plant. Physiol.* 107:1257-1268; Li *et al.*, 1995, *Plant Physiol.* 109:117-185). (iii) DNP-GS is the only known immediate metabolite of CDNB *in vivo* (Li *et al.*, 1995, *Plant Physiol.* 109:117-185).

15 Methods similar to those described by Howden *et al.* (Howden *et al.*, 1992, *Plant Physiol.* 99:100-107; Howden *et al.*, 1995, *Plant Physiol.* 107:1059-1066; Howden *et al.*, 1995, *Plant Physiol.* 107:1067-1073) were applied to the initial characterization of the transformants. T2 seeds were first sown in rows on Cd²⁺-free and CDNB-free medium in Petri dishes standing on edge so that the roots grew vertically down the surface of the agar. Three to 4 days after germination, the seeds were transferred, again in rows, to media containing a range of CdSO₄ or CDNB concentrations. After rotating the Petri dishes though 180° and allowing growth for another 24-48 hours, the seedlings were scored for hook length. The results of this study are shown in Figure 28. It is evident from the data presented therein that transgenic *Arabiposis* plants comprising *YCF1* acquire increased resistance to cadmium salts and the organic cytotoxin, CDNB.

25 The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific
embodiments, it is apparent that other embodiments and variations of this invention
may be devised by others skilled in the art without departing from the true spirit and
scope of the invention. The appended claims are intended to be construed to include all
5 such embodiments and equivalent variations.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rea, Philip A
Lu, Yu-Ping
Li, Ze-Sheng
- (ii) TITLE OF INVENTION: GLUTATHIONE-S-CONJUGATE TRANSPORT IN
PLANTS
- (iii) NUMBER OF SEQUENCES: 20
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 - (E) COUNTRY: US
 - (F) ZIP: 19103-7086
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: Not yet assigned
 - (B) FILING DATE: 18-NOV-1997
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/031,040
 - (B) FILING DATE: 18-NOV-1996
- (viii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 60/061,328
 - (B) FILING DATE: 08-OCT-1997
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5232 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 9936 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CCTCTCTTTT ATTCATATGA TCACAGGTTT TCGTCATGT TGGATAACCT TCTGTCACGT 2400
GGAAGTCATT TATAATTAC ATGGTGTTAC AGATTATTAG AAGGAACTAG TGGGTTCTTA 2460
GTTTTCTTT ATCAATTCAT TGTACTTGAA CATATTTATT TACATTTGTA TGCACAGATC 2520
GGGAATGATT GCTCACAGTT TGTGGGACCT CTTTACTGA ATCAACTCTT AAAGGTTTGT 2580
TCTTTTCTTG GCAGATTCGG AAACCTATTA TTGGTTCAAT ATTCTTATCT GACAATATCT 2640
CTCATTTTGG ATGTCAAAT ATATACAGTC AATGCAAGAG GATGCGCCAG CTGGATGGG 2700
TTACATCTAT GCGTCTCAA TCTTTGGTGG AGTGGTATGA AATGAAGTCC TCTTCTCTC 2760
TCTCTCTCTG TCTATTGGA CTCTCTTCTA TCAACTGTG AACTGACAC TTGTTATACT 2820

TCGTATGTT TGGTCTAAGG TTCTTCTAAA CTGATTATAA TAGCAACACT AGATGTCCCC	2880
TAATGCCACT TTTTGATTTT GTTGCTCTTG GATTTTTTGC GTCTGTTAGA TAGGTTCTGA	2940
CTTTATCTAG TGTAGGGTGA TACTTAAAGC TACAAACTCA TCGAGTGA CTGATGTTGATG	3000
ACAACGTTTC TAGGTGTTCTG GGGTGCTATG TGAAGCTCAA TATTTCCAGA ATGTCATGCG	3060
TGTTGGTTAC CGACTGAGAT CTGCTCTGGT AAATTTTAAA TTTGCTACCC TGACGTTCTT	3120
CCTTTGCCAT ATGTTTTTGG TGCAGATATG TTTGCTGATA GCATGATTCC CAGTATCTTG	3180
TATAGGAATA AGTATATCAA CATGGTTTCT TTATCCTCTA TATATGATGC ATAAATAAGC	3240
CTTGTCCTAA AAGTTTAGGA ATAAGTTTGT GTTGCTTCAG ATGATTGAGT ATGCTGTTTT	3300
TATTTCTGGA AATTTCCACC ATTTTCAGAT CCTTCTACTA GAGAAATACA AATTTAGCTG	3360
TATTTCTGGA TTCAGTTCAT CGTTTTCTGC GTTTGTAGTG GAGTGAAATT AGCTTGTACG	3420
AAATGGAAGA TATTTTGAAC ACAGATGATT TTTAAAATTG GTCTTCCTGT TGATGACTGT	3480
TTTTTTTTTA GATTGCTGCT GTGTTCCGCA AATCGTTGAG GTTAACTAAT GAAGGTCGTA	3540
GAAAGTTTCA AACAGGAAAG ATAACCAACT TAATGACGAC TGATGCCGAA TCTCTTCAGG	3600
TGAGTATCCC TTTCATATTT TCGAATTCAA GTTTGCATGT TTCTCTATAT CATAGTTGCA	3660
GGGCTGTAA CATCCGGATC TTGAATATTT ATTTTGTGCC GCAGCTGGTA TTGAGTGGGT	3720
TACAGTTACT TTTTATGTTT GGTAATAGAA GTTGGATTTA CTTAGAAATG ATTTCCAGCA	3780
TACTGATCTA CTGAATCTGT TTGTTAGGTC TAAGATTGGC TATGAATAGT GATTGCATTT	3840
TCATTTCTAG CTAGCACTTT GTTATCATTG AATTTTTCTT TCTTCTTTT TATTTTGTIT	3900
CTTATGCCAA CTTAAACTGT GTCTTGTTTA ATGTTTTCTG CTTAACTGTG TCTGGTATCA	3960
ATATTGTTAT CTAATCAACC AGATGTACTT TGTACTAATT TTTCCATTTT CTGTGGCAGC	4020
AAATATGCCA ATCACTTCAT ACCATGTGGT CGGCTCCATT TCGTATAATT ATAGCACTGA	4080
TTCTCCTCTA TCAGCAATTG GGTGTTGCCT CGCTCATTGG TGCATTGTTG TTGGTCCTTA	4140
TGTTCCCTTT ACAGGTACAT GACTTCTAAA TTCTCTCATT TTTTTCCTT TGTAGCTTAT	4200
TTTTCTCTAT ACTGTTGCTT TGTTCAATCG TACTCCTAAA GGCTACTTCT TCTTCGTCTC	4260
CTGAACTTGT TCTCTGTTTT CTTAAACAG ACTGTTATTA TAAGCAAAAT GCAGAAGCTG	4320

ACAAAGGAAG GTCTGCAGCG TACTGACAAG AGAATTGGCC TTATGAATGA AGTTTTAGCT 4380
GCAATGGATA CAGTAAAGTA AGAAATTCTA GAACCAATTT TGTTAACATA GTTATTAATT 4440
TGCAGGAAAC TTGTACTAAA CCAAATGCT ACAGGTGTTA TGCTTGGGAA AACAGTTTCC 4500
AGTCCAAGGT CCAAAGTGC GTGATGATGA ATTATCTTGG TTCCGGAAAT CACAGCTCCT 4560
GGGAGCGGTA TGACTACAGC GTAGTTACTT TTGTTTTTCC TCTAATTATT GTATATTCT 4620
AACTCTTGCT TGGTCTTGTC TTGTTTTGCA GTTGAATATG TTCATACTGA ATAGCATTCC 4680
TGTCTTGTG ACTATTGTTT CATTGGTGT GTTCACATTA CTTGGAGGAG ACCTGACCCC 4740
TGCAAGAGCA TTTACGTCAC TCTCTCTCTT TGCTGTGCTT CGTTTCCCTC TCTTCATGCT 4800
TCCAAACATT ATAAGTCAAG TGATTCTTA AATATGTTGT TGCAATGCAT GTGTATTAAG 4860
TAGAACTGTT AGTGCTTGTA GTAAGTGTG TTTGGTTATC AAATCCATGA CTTATATTTT 4920
GAATTTACAT GCTGGAGGGT ATCCTTGCTG GTGCCAGAAA CAGATGCCGA TGCTGACTAG 4980
TTTTCACTTG TAGGTGGTAA ATGCTAATGT ATCCTTAAAA CGTCTTGAGG AGGTATTGGC 5040
GACAGAAGAA AGAATTCTCT TACCAAATCC TCCCATGAA CCTGGAGAGC CAGCCATCTC 5100
AATAAGAAAT GGATATTTCT CTTGGGATTC TAAGGTGTG CTTGGCTATT CTATACCATG 5160
TTCCTTCTTT CGCTTCTCTC ATTACCTTTA TCCATAGAAA GTACAAAAAT CGAGCTAACC 5220
CTATGTATCT ACAGGGGGAT AGGCCGACGT TGTCAAATAT CAACTGGAT GTACCTCTTG 5280
GCAGCCTAGT TGCTGTGGTT GGTAGTACAG GCGAAGGAAA AACCTCTCTA ATATCTGCTA 5340
TCCTTGGTGA ACTTCTGCA ACATCTGATG CAATAGTTAC TCTCAGAGGA TCAGTTGCTT 5400
ATGTTCCACA AGTTTCATGG ATCTTTAATG CAACAGTATG TTCTTCTTTT CTTTGACTTT 5460
TAAGTTGGGC TGACGTGCA AATTTTCTG TTGTACATAA TGTTAAATGT ATTTTCTGTC 5520
TTTTATAGTA GAACAATATG TGTTCTCAA TCGGTCAGTT ACTTCACCAA CTTAGTGGAA 5580
ACCTTCTTCA ATATTTGATT CTCTAAGCTA TTTGAACAG AAGACTGATA TGCATTTTCT 5640
TATAAAATT TGTAGGTACG CGACAATATA CTGTTGGTT CTCCTTTCGA CCGTGAAAAG 5700
TATGAAAGGG CCATTGATGT GACTTCACTG AAGCATGACC TAGAGTTACT GCCTGTAAGT 5760
TTTGAGGAGA GCTTCGTGGA GTTGATAACA AGGATTTGTC TTGCCTGTTT TCGTGTGCT 5820

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AAGAGGTGTT AATATCAGTG GAGGACAGAA GCAGAGGGTT TCCATGGCTA GGGCCGTTTA	5940
CTCAAATTCA GATGTGTACA TCTTTGATGA CCCGTTAAGT GCCCTTGATG CTCATGTTGG	6000
TCAACAGGTA CTAACCTATT GATTCTCTTT GATAAGGCTA GTCTATTTCA TTTTGAATT	6060
TATCTAACAT TTTTGTGTCT GGTCATTATG GGAATACTGT CAGTCTGATT TCTAGGAATA	6120
TTGTTTCAGG TTTTGTAAAA ATGCATAAAA AGAGAACTGG GGCAGAAAAC GAGAGTTCTT	6180
GTTACAAACC AGCTCCACTT CCTATCACAA GTGGACAGAA TTGTACTTGT GCATGAAGGC	6240
ACAGTGAAAG AGGAAGGAAC ATATGAAGAG CTATCCAGTA ATGGGCCTTT GTTCCAGAGG	6300
GTAATGGAAG ATGCAGGGAA GGTGGAAGAA TATTCAGAAG AAAATGGAGA AGCTGAGGCA	6360
GACCAAACAG CGGAACAACC AGTTGCGAAT GGGAAACAAA ATGGTCTTCA AATGGATGGA	6420
AGTGACGATA AAAAATCCAA AGAAGGAAAT AAAAAAGGAG GGAAATCTGT CCTCATCAAG	6480
CAAGAAGAAC GTGAAACCGG AGTTGTAAGT TGGAGAGTCC TGAAGAGGTA ACTTGAACAT	6540
TTGGCTTTTG CAATCTTACT ATTTGTTTGC AACTTTCCCC ATACTCGATC CAAGAGGTCC	6600
ATTCAATTGT GGTGTTTCAC AACAACTAG CATGTTCCCTT ATGTTTTTAG GCTGAACAT	6660
ACCTTTGCGG GATATCAGAA TGACTTTTCC AGGCTTTCAA TGTTTTCAGG TACCAGGATG	6720
CACTTGAGG GGCATGGGTA GTGATGATGC TCCTTTTATG TTACGTCTTA ACAGAAGTAT	6780
TTGCGGTAC TAGCAGCAG TGGTTGAGTG AGTGGACTGA TGCAGGAAC CCAAAGAGTC	6840
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TGTTTGCTTG ATGGATGAGT GAAGATTTGA TATAATCTTG ACCTCATGAT ATAACATATA	6960
TAGCTGAAAC CTGACCAGCT TAGAAAGATC TTATATAATT CTACTTTTGT GATTTTACTT	7020
TGAGAATCCA AAGGTGGAGG TAGAAAAGGT TAGTAAAGAA TTGATTTTTT TGCTGAGACT	7080
CTTCTTCTT GCTTACAGG TTTGGTGACA TTGACCAATT CATATTGGTT GATTATGTCC	7140
AGTCTTTATG CAGCTAAGAA GTTACACGAC AATATGCTTC ATTCCATACT GAGGGCCCCG	7200
ATGTCTTCT TCCATACCAA TCCGCTAGGA CGGATAATCA ATCGATTGCG AAAAGATCTG	7260
GGTGATATTG ATCGAACTGT GGCCGTCTTT GTAAACATGT TTATGGGTCA AGTCTCACAG	7320

CTTCTTTCAA CTGTAGTGTT GATTGGCATT GTAAGCACTT TGTCTTGTG GGCCATCATG 7380
CCCCCTCTGG TCTTGTTTTA TGGAGCTTAT CTTTATTATC AGGTAATGTA CCTTCTGACC 7440
GCAGCATTTA AATAACTGAG ATTAAGTGAC AGAAAGAGAA AAGGACACAG ATGATGGATG 7500
TTACACATAC TTTTITAGCC TCATTTGTCA TGTCTGAGTT CGTTTGGTGC TTAAGCTATC 7560
TACACTCATC TGTACCAAAA AATCATGCTG TATATGTTGT GTGTTAAATA TTTTCTTAT 7620
TGCAGAACAC AGCCCGTGAG GTTAAGCGTA TGGATTCAAT TTCAAGATCG CCTGTTTATG 7680
CACAGTTTGG AGAGGCATTG AATGGCTTAT CAACTATCCG TGCTTACAAA GCATATGATC 7740
GTATGGCTGA TATCAACGGA AGATCAATGG ATAATAACAT CAGATTCACCT CTTGTCAACA 7800
TGGGTGCCAA TCGGTGGCTT GGAATCCGTT TAGAACTCT GGGTGGTCTT ATGATATGGC 7860
TGACAGCATC GTTGCTGTC ATGCAGAATG GAAGAGCGGA GAACCAACAG GCATTTGCAT 7920
CTACAATGGG TTTGCTTCTC AGTTATGCCT TAAATATTAC TAGCTTGTTA ACAGGTGTTT 7980
TGAGACTTGC GAGTTTGGCT GAGAATAGTC TAAACGCGGT CGAGTGTGG CAATTATATA 8040
GAGATTCCGC CAGAGGTCCG CCTGTCATTG AGAACAACCG TCCACCTCCT GGATGGCCAT 8100
CATCTGGATC CATAAAGTTT GAGGATGTG TTCTCCGTTA CCGCCCTCAG TTACCGCCTG 8160
TGCTTCATGG GGTTCCTTC TTCATTCATC CAACAGATAA GGTGGGGATT GTTGAAGGA 8220
CTGGTGCTGG AAAGTCAAGC CTGTTGAATG CATTGTTTAG AATTGTGGAG GTGGAAAAAG 8280
GAAGGATCTT AATCGATGAT TGTGACGTTG GAAAGTTTGG ACTGATGGAC CTACGTAAAG 8340
TGCTCGGAAT CATTCACAG TCACCGGTTT TTTCTCAGG AACTGTGAGG TTCAATCTTG 8400
ATCCATTTGG TGAACACAAT GATGCTGATC TTTGGGAATC TCTAGAGAGG GCACACTTGA 8460
AGGATACCAT CCGCAGAAAT CCTCTGGTC TTGATGCTGA GGTATTCAGT TGCTGCCTAT 8520
ATTGATATGA AGTCTCATTT TTTAAGTGGT AATAACTGAT TTTCAATCTT TGTTCAGGTC 8580
TCTGAGGCAG GAGAGAATTT CAGCGTGGGA CAGAGGCAAT TGTGAGTCT TTCACGTGCG 8640
CTGTTACGGA GATCTAAGAT ACTCGTCCTT GATGAAGCAA CTGCTGCTG AGATGTTAGA 8700
ACCGATGCCC TCATTCAGAA GACTATCCGA GAAGAATTCA AGTCATGCAC GATGCTCATT 8760
ATCGCTCACC GTCTCAATAC CATCATTGAC TGTGACAAAA TTCTCGTGCT TGATTCTGGA 8820

AGAGTATGAT TTTAAACACT CTCTCTCTTT CAATCTCACA CTCTCCTTGT TTCTCAGCTA	8880
ACCTGTTCTA TTCCAATTG TTAACCTCAGG TTCAAGAATT CAGTTCACCG GAGAACCTTC	8940
TTTCAAATGA AGGAAGCTCT TTCTCCAAGA TGGTTCAAAG CACTGGAGCT GCAAATGCTG	9000
AGTACTTGCG TAGTTTAGTA CTCGACAACA AGCGTGCCAA AGATGACTCA CACCACTTAC	9060
AAGGCCAAAG GAAATGGCTG GCTTCTTCTC GCTGGGCTGC AGCCGCTCAG TTTGCTCTGG	9120
CTGCGAGTCT TACTTCGTCG CACAACGATC TTCAAAGCCT TGAAATTGAA GATGACAGCA	9180
GCATTTTGAA GAGAACAAAC GATGCAGTTG TGA CTCTGCG CAGTGTCTC GAGGGGAAAC	9240
ACGACAAAGA GATTGCAGAG TCGCTTGAGG AACATAATAT CTCTAGAGAG GGATGGTTGT	9300
CATCACTCTA TAGAATGGTA GAAGGTAAAC CAAATATGCA TCTCTACAAA TGCTTATGCA	9360
AAATCTTAAT CACCACACTG AAACATTAAA GTCAAATCGT GCTCTTATAT TGCAAGCCTG	9420
CTTTCGCTG TCTACGTTTC AGGGCTTGCA GTGATGAGCA GATTGGCAAG GAACCGAATG	9480
CAACAACCGG ATTACAATTT CGAAGGAAAT ACATTTGACT GGGACAACGT CGAGATGTAG	9540
ATAAGTTCAT GTTAACTAG GAATCATTGT CTCTCCGTA AGAAACATAT ATTTATCTTA	9600
ACCAAATTA TTAGTTTGGT TTCCATTTCA TAACTTAAT TTTCACCTGC AAAGAAAATC	9660
AAACCCTGTT GTGTTCTTCG TGATAAGTAG AGAAATTA CTGAGTATCCT TCTAACTCAT	9720
AAATGGGATC TCATGATTCA TGAACAAGCA GCAACACAAT AATACCCTTT TCAGATTTTG	9780
GAGCTGGACA AAGTTGTAA GTTGAGTTTC TCTTACAGTC ATTCATATAC AAAAACCTCT	9840
TCGACTGAAG CACCAAGAAA GAAACAAACA TCAAAGGGA ATGAGGTCTT TTCTTAGGGC	9900
TGAGATCATC GGAATGTGGG AGTGCGGAAC ACGACC	9936

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1621 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

Met Gly Phe Glu Phe Ile Glu Trp Tyr Cys Lys Pro Val Pro Asn Gly
1 5 10 15

Val Trp Thr Lys Thr Val Ala Asn Ala Phe Gly Ala Tyr Thr Pro Cys
20 25 30

Ala Thr Asp Ser Phe Val Leu Gly Ile Ser Gln Leu Val Leu Val
35 40 45

Leu Cys Leu Tyr Arg Ile Trp Leu Ala Leu Lys Asp His Lys Val Glu
50 55 60

Arg Phe Cys Leu Arg Ser Arg Leu Tyr Asn Tyr Phe Leu Ala Leu Leu
65 70 75 80

Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Ile Met Gly Ile
85 90 95

Ser Val Leu Asp Phe Asp Gly Pro Gly Leu Pro Pro Phe Glu Ala Phe
100 105 110

Gly Leu Gly Val Lys Ala Phe Ala Trp Gly Ala Val Met Val Met Ile
115 120 125

Leu Met Glu Thr Lys Ile Tyr Ile Arg Glu Leu Arg Trp Tyr Val Arg
130 135 140

Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
145 150 155 160

Val Leu Ser Val Lys Glu Tyr Tyr Ser Ser Tyr Val Leu Tyr Leu Tyr
165 170 175

Thr Ser Glu Val Gly Ala Gln Val Leu Phe Gly Ile Leu Leu Phe Met
180 185 190

His Leu Pro Asn Leu Asp Thr Tyr Pro Gly Tyr Met Pro Val Arg Ser
195 200 205

Glu Thr Val Asp Asp Tyr Glu Tyr Glu Glu Ile Ser Asp Gly Gln Gln
210 215 220

Ile Cys Pro Glu Lys His Pro Asn Ile Phe Asp Lys Ile Phe Phe Ser
225 230 235 240

Trp Met Asn Pro Leu Met Thr Leu Gly Ser Lys Arg Pro Leu Thr Glu
245 250 255

Lys Asp Val Trp Tyr Leu Asp Thr Trp Asp Gln Thr Glu Thr Leu Phe
 260 265 270
 Thr Ser Phe Gln His Ser Trp Asp Lys Glu Leu Gln Lys Pro Gln Pro
 275 280 285
 Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp
 290 295 300
 Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro
 305 310 315 320
 Leu Leu Leu Asn Gln Leu Leu Lys Ser Met Gln Glu Asp Ala Pro Ala
 325 330 335
 Trp Met Gly Tyr Ile Tyr Ala Phe Ser Ile Phe Gly Gly Val Val Phe
 340 345 350
 Gly Val Leu Cys Glu Ala Gln Tyr Phe Gln Asn Val Met Arg Val Gly
 355 360 365
 Tyr Arg Leu Arg Ser Ala Leu Ile Ala Ala Val Phe Arg Lys Ser Leu
 370 375 380
 Arg Leu Thr Asn Glu Gly Arg Arg Lys Phe Gln Thr Gly Lys Ile Thr
 385 390 395 400
 Asn Leu Met Thr Thr Asp Ala Glu Ser Leu Gln Gln Ile Cys Gln Ser
 405 410 415
 Leu His Thr Met Trp Ser Ala Pro Phe Arg Ile Ile Ile Ala Leu Ile
 420 425 430
 Leu Leu Tyr Gln Gln Leu Gly Val Ala Ser Leu Ile Gly Ala Leu Leu
 435 440 445
 Leu Val Leu Met Phe Pro Leu Gln Thr Val Ile Ile Ser Lys Met Gln
 450 455 460
 Lys Leu Thr Lys Glu Gly Leu Gln Arg Thr Asp Lys Arg Ile Gly Leu
 465 470 475 480
 Met Asn Glu Val Leu Ala Ala Met Asp Thr Val Lys Cys Tyr Ala Trp
 485 490 495
 Glu Asn Ser Phe Gln Ser Lys Val Gln Thr Val Arg Asp Asp Glu Leu
 500 505 510
 Ser Trp Phe Arg Lys Ser Gln Leu Leu Gly Ala Leu Asn Met Phe Ile
 515 520 525

Leu Asn Ser Ile Pro Val Leu Val Thr Ile Val Ser Phe Gly Val Phe
 530 535 540

Thr Leu Leu Gly Gly Asp Leu Thr Pro Ala Arg Ala Phe Thr Ser Leu
 545 550 555 560

Ser Leu Phe Ala Val Leu Arg Phe Pro Leu Phe Met Leu Pro Asn Ile
 565 570 575

Ile Thr Gln Val Val Asn Ala Asn Val Ser Leu Lys Arg Leu Glu Glu
 580 585 590

Val Leu Ala Thr Glu Glu Arg Ile Leu Leu Pro Asn Pro Pro Ile Glu
 595 600 605

Pro Gly Glu Pro Ala Ile Ser Ile Arg Asn Gly Tyr Phe Ser Trp Asp
 610 615 620

Ser Lys Gly Asp Arg Pro Thr Leu Ser Asn Ile Asn Leu Asp Val Pro
 625 630 635 640

Leu Gly Ser Leu Val Ala Val Val Gly Ser Thr Gly Glu Gly Lys Thr
 645 650 655

Ser Leu Ile Ser Ala Ile Leu Gly Glu Leu Pro Ala Thr Ser Asp Ala
 660 665 670

Ile Val Thr Leu Arg Gly Ser Val Ala Tyr Val Pro Gln Val Ser Trp
 675 680 685

Ile Phe Asn Ala Thr Val Arg Asp Asn Ile Leu Phe Gly Ser Pro Phe
 690 695 700

Asp Arg Glu Lys Tyr Glu Arg Ala Ile Asp Val Thr Ser Leu Lys His
 705 710 715 720

Asp Leu Glu Leu Leu Pro Gly Gly Asp Leu Thr Glu Ile Gly Glu Arg
 725 730 735

Gly Val Asn Ile Ser Gly Gly Gln Lys Gln Arg Val Ser Met Ala Arg
 740 745 750

Ala Val Tyr Ser Asn Ser Asp Val Tyr Ile Phe Asp Asp Pro Leu Ser
 755 760 765

Ala Leu Asp Ala His Val Gly Gln Gln Val Phe Glu Lys Cys Ile Lys
 770 775 780

Arg Glu Leu Gly Gln Lys Thr Arg Val Leu Val Thr Asn Gln Leu His
 785 790 795 800

Phe Leu Ser Gln Val Asp Arg Ile Val Leu Val His Glu Gly Thr Val
 805 810 815
 Lys Glu Glu Gly Thr Tyr Glu Glu Leu Ser Ser Asn Gly Pro Leu Phe
 820 825 830
 Gln Arg Leu Met Glu Asn Ala Gly Lys Val Glu Glu Tyr Ser Glu Glu
 835 840 845
 Asn Gly Glu Ala Glu Ala Asp Gln Thr Ala Glu Gln Pro Val Ala Asn
 850 855 860
 Gly Asn Thr Asn Gly Leu Gln Met Asp Gly Ser Asp Asp Lys Lys Ser
 865 870 875 880
 Lys Glu Gly Asn Lys Lys Gly Gly Lys Ser Val Leu Ile Lys Gln Glu
 885 890 895
 Glu Arg Glu Thr Gly Val Val Ser Trp Arg Val Leu Lys Arg Tyr Gln
 900 905 910
 Asp Ala Leu Gly Gly Ala Trp Val Val Met Met Leu Leu Leu Cys Tyr
 915 920 925
 Val Leu Thr Glu Val Phe Arg Val Thr Ser Ser Thr Trp Leu Ser Glu
 930 935 940
 Trp Thr Asp Ala Gly Thr Pro Lys Ser His Gly Pro Leu Phe Tyr Asn
 945 950 955 960
 Leu Ile Tyr Ala Leu Leu Ser Phe Gly Gln Val Leu Val Thr Leu Thr
 965 970 975
 Asn Ser Tyr Trp Leu Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Leu
 980 985 990
 His Asp Asn Met Leu His Ser Ile Leu Arg Ala Pro Met Ser Phe Phe
 995 1000 1005
 His Thr Asn Pro Leu Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Leu
 1010 1015 1020
 Gly Asp Ile Asp Arg Thr Val Ala Val Phe Val Asn Met Phe Met Gly
 1025 1030 1035 1040
 Gln Val Ser Gln Leu Leu Ser Thr Val Val Leu Ile Gly Ile Val Ser
 1045 1050 1055
 Thr Leu Ser Leu Trp Ala Ile Met Pro Leu Leu Val Leu Phe Tyr Gly
 1060 1065 1070

Ala Tyr Leu Tyr Tyr Gln Asn Thr Ala Arg Glu Val Lys Arg Met Asp
 1075 1080 1085

Ser Ile Ser Arg Ser Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn
 1090 1095 1100

Gly Leu Ser Thr Ile Arg Ala Tyr Lys Ala Tyr Asp Arg Met Ala Asp
 1105 1110 1115 1120

Ile Asn Gly Arg Ser Met Asp Asn Asn Ile Arg Phe Thr Leu Val Asn
 1125 1130 1135

Met Gly Ala Asn Arg Trp Leu Gly Ile Arg Leu Glu Thr Leu Gly Gly
 1140 1145 1150

Leu Met Ile Trp Leu Thr Ala Ser Phe Ala Val Met Gln Asn Gly Arg
 1155 1160 1165

Ala Glu Asn Gln Gln Ala Phe Ala Ser Thr Met Gly Leu Leu Leu Ser
 1170 1175 1180

Tyr Ala Leu Asn Ile Thr Ser Leu Leu Thr Gly Val Leu Arg Leu Ala
 1185 1190 1195 1200

Ser Leu Ala Glu Asn Ser Leu Asn Ala Val Glu Arg Val Gly Asn Tyr
 1205 1210 1215

Ile Glu Ile Pro Pro Glu Ala Pro Pro Val Ile Glu Asn Asn Arg Pro
 1220 1225 1230

Pro Pro Gly Trp Pro Ser Ser Gly Ser Ile Lys Phe Glu Asp Val Val
 1235 1240 1245

Leu Arg Tyr Arg Pro Gln Leu Pro Pro Val Leu His Gly Val Ser Phe
 1250 1255 1260

Phe Ile His Pro Thr Asp Lys Val Gly Ile Val Gly Arg Thr Gly Ala
 1265 1270 1275 1280

Gly Lys Ser Ser Leu Leu Asn Ala Leu Phe Arg Ile Val Glu Val Glu
 1285 1290 1295

Glu Gly Arg Ile Leu Ile Asp Asp Cys Asp Val Gly Lys Phe Gly Leu
 1300 1305 1310

Met Asp Leu Arg Lys Val Leu Gly Ile Ile Pro Gln Ser Pro Val Leu
 1315 1320 1325

Phe Ser Gly Thr Val Arg Phe Asn Leu Asp Pro Phe Gly Glu His Asn
 1330 1335 1340

Asp Ala Asp Leu Trp Glu Ser Leu Glu Arg Ala His Leu Lys Asp Thr
 1345 1350 1355 1360
 Ile Arg Arg Asn Pro Leu Gly Leu Asp Ala Glu Val Ser Glu Ala Gly
 1365 1370 1375
 Glu Asn Phe Ser Val Gly Gln Arg Gln Leu Leu Ser Leu Ser Arg Ala
 1380 1385 1390
 Leu Leu Arg Arg Ser Lys Ile Leu Val Leu Asp Glu Ala Thr Ala Ala
 1395 1400 1405
 Val Asp Val Arg Thr Asp Ala Leu Ile Gln Lys Thr Ile Arg Glu Glu
 1410 1415 1420
 Phe Lys Ser Cys Thr Met Leu Ile Ile Ala His Arg Leu Asn Thr Ile
 1425 1430 1435 1440
 Ile Asp Cys Asp Lys Ile Leu Val Leu Asp Ser Gly Arg Val Gln Glu
 1445 1450 1455
 Phe Ser Ser Pro Glu Asn Leu Leu Ser Asn Glu Gly Ser Ser Phe Ser
 1460 1465 1470
 Lys Met Val Gln Ser Thr Gly Ala Ala Asn Ala Glu Tyr Leu Arg Ser
 1475 1480 1485
 Leu Val Leu Asp Asn Lys Arg Ala Lys Asp Asp Ser His His Leu Gln
 1490 1495 1500
 Gly Gln Arg Lys Trp Ala Ser Ser Arg Trp Ala Ala Ala Ala Gln Phe
 1505 1510 1515 1520
 Ala Leu Ala Ala Ser Leu Thr Ser Ser His Asn Asp Leu Gln Ser Leu
 1525 1530 1535
 Glu Ile Glu Asp Asp Ser Ser Ile Leu Lys Arg Thr Asn Asp Ala Val
 1540 1545 1550
 Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ala Glu
 1555 1560 1565
 Ser Leu Glu Glu His Asn Ile Ser Arg Glu Gly Trp Leu Ser Ser Leu
 1570 1575 1580
 Tyr Arg Met Val Glu Gly Leu Ala Val Met Ser Arg Leu Ala Arg Asn
 1585 1590 1595 1600
 Arg Met Gln Gln Pro Asp Tyr Asn Phe Glu Gly Asn Thr Phe Asp Trp
 1605 1610 1615

Asp Asn Val Glu Met
1620

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5175 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCGCGG CCGCCGGCGA ATTTGCACTC TTTACCTCTC TTTGACTCCG TGAGATTCTGA	60
GGATTGTTAG TTTCTTGTA TGTGTAGTCT TTGAAGCAGG GGATTTTAT TGTATTGAGG	120
AAGAAGATGG GGTTCGAGCC GTTGGATTGG TATTGCAAGC CGGTGCCGAA TGGTGTGTGG	180
ACTAAACTG TGGATTATGC GTTGGTGCA TACACGCCTT GTGCTATTGA CTCTTTGTG	240
CTTGGTATCT CTCATCTGGT TCTGTTGATT CTGTGTCTTT ATCGCTGTG GCTCATCAG	300
AAGGATCACA AAGTGGATAA GTTCTGCTTG AGGTCTAAAT GGTTTAGCTA TTTTCTGGCT	360
CTTTTGGCTG CTTATGCTAC TCGGAGCCT TTGTTTAGAT TGGTCATGAG GATCTCTGTT	420
TTGGATTGG ATGGAGCTGG GTTCCTCCC TATGAGGCGT TTATGTTGGT CCTTGAGGCT	480
TTTGCTTGGG GTTCTGCTTT GGTCACTACT GTTGTGGAAA CTAACGTA TATCCATGAA	540
CTCCGTTGGT ATGTCAGATT CGCTGTCATT TATGCTCTTG TGGAGACAT GGTGTTGTTA	600
AATCTTGTTT TCTCTGTTAA GGAGTACTAT GGCAGTTTTA AACTGTATCT TTACATAAGC	660
GAGGTGGCAG TTCAGGTTGC ATTTGGAACC CTCTGTTTG TGTATTTCCC TAATTGGAC	720
CCTTACCCTG GTTACACACC AGTTGGGACT GAAAATCCG AGGATTACGA GTATGAAGAG	780
CTTCCTGGAG GAGAAAATAT ATGTCCTGAG AGGCATGCAA ATTTATTGTA CAGTATCTTC	840
TTCTCATGGT TGAACCCATT GATGACTCTG GGATCAAAAC GACCTCTCAC CGAGAAGGAT	900
GTATGGCATC TGGACACTTG GGATAAACT GAACTCTTA TGAGGAGCTT CCAGAAGTCC	960

TGGGATAAGG AACTAGAAAA GCCCAAACCG TGGCTTTTGA GAGCACTGAA CAACAGCCTT	1020
GGGGGAAGGT TTTGGTGGGG TGGCTTTTGG AAGATTGGGA ATGACTGTTC ACAGTTCGTG	1080
GGGCCTCTTC TACTGAATGA GCTCTTAAAG TCAATGCAAC TTAATGAACC AGCGTGGATA	1140
GGTTACATCT ATGCAATCTC AATCTTTGTT GGAGTGGTAT TGGGGGTTTT ATGTGAAGCT	1200
CAGTATTTC AAAATGTGAT GCGTGTGGT TACCGGCTTA GGTCTGCACT GATTGCTGCT	1260
GTGTTCCGAA AATCTTTGAG GCTAACTAAT GAGGGGCGGA AGAAGTTTCA AACAGGAAAA	1320
ATAACAACT TAATGACTAC TGATGCTGAG TCGCTGCAGC AAATCTGCCA ATCACTTCAT	1380
ACCATGTGGT CGGCGCCATT TCGTATAATT GTAGCACTGG TTCTCCTCTA TCAACAATTG	1440
GGTGTTCCT CGATCATTGG TGCATTGTTT CTTGTCCTTA TGTTCCTCAT ACAGACTGTT	1500
ATTATAAGCA AAACGCAGAA GTTAACAAAA GAAGGGTTGC AGCGTACTGA CAAGAGAATT	1560
GGCCTAATGA ATGAGGTTTT AGCGGCAATG GATACAGTGA AGTGTTACGC TTGGGAAAAC	1620
AGTTTTCAGT CCAAGGTTCA AACTGTACGT GATGATGAAT TATCTTGGTT CCGGAAAGCA	1680
CAACTCCTGT CAGCGTTCAA TATGTTTATA CTAAACAGCA TCCCTGTCCT CGTGACTGTT	1740
GTTTCATTGG GTGTGTTCTC ATTGCTTGA GGAGATCTGA CACCTGCAAG AGCGTTTACG	1800
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GATACTTCTC CTGGGATTCA AAGGCGGATA GGCCAACATT GTCAAACATC AACCTGGACA	2040
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TATCTGCTAT GCTTGGGGAA CTTCTGCAA GATCTGATGC GACTGTTACT CTTAGAGGAT	2160
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TGTTTGGGGC TCCTTTTGAC CAAGAAAAAT ATGAAAGGGT GATTGATGTG ACAGCACTCC	2280
AGCATGACCT TGAGTTACTG CCTGGAGGTG ACCTCACGGA GATCGGAGAA AGGGGTGTTA	2340
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CGCTTCTTTC GTTTGGACAG GTCTCTGTGA CATTGATCAA TTCATATTGG TTGATTATGT	3060
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GGTCAATGGA CAATAACATC AGATTCACAC TTGTAAACAT GGCTGCAAAT CGGTGGCTGG	3540
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TGCAGAACGG AAAGGCAGCG AACCAACAAG CATATGCATC TACGATGGGT TTGCTTCTCA	3660
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ACGACGCCGA TCTCTGGGAA TCTCTTGAGA GGGCACACTT GAAAGATACT ATCCGCAGAA	4200
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AGCGTTGGTG GCCATCTCTT TACAAAATGG TCGAAGGGCT TGCCGTGATG AGCAGATTGG	4920
CGAGGAACAG AATGCAACAC CCGGATTACA ATTTAGAAGG GAAATCGTTT GACTGGGACA	4980
ATGTCGAGAT GTAAACGATG AAAGGCTTAC ACTAATAGAC CTAAAACTCC CATTTTGATG	5040
GAACTTTTAT TTGTATTGCT TGGGATACAC GTAACAAAAT GCCCATTAAT CGTGGTGTA	5100
CTATATAGGC TATGCTTCTT TTGGGAAAAA GAGAGTTTGA TTACAGAGGA TGTGATGATA	5160
ACACAATTGG AATTC	5175

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10342 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGGAGGTTTG GTTTTTTCCC TATCAATCGA ATTCCATTTC GTGCTCGTAA CGTGGATTTT	60
GGTAGATTTT TTTTAGGGGG ATGGAACTT GTTTATTATC TATAGATGAT GATTTTGTTT	120
TCTCCATGAG AATGTATGCT TTAAACTTT TTTTTTTTG TTTTTCCT TCGGAGCTAA	180
CTTTGGGGGC TGGTCTCGGT CTCTGTTTC TCTCCACTAA AAAGATAAAA AGCTTTTGCC	240
ATCTTTTTT TTTTCTCAAT AATCTATCAC ATCGTTTTT TTCTTTGTT TTTTCTCCAT	300
TTGTCTTCAT TGAGTTCATA GCCACATAAT TATTGATTTC TTTTCTTTT AGTGTTCCTG	360
TTACTGATGC GTTTCATTAT TTATACTTCT CACTTGCAGA TCGAGGATT GTTAGTTTCT	420
TGTGATGTGT AGTCTTTGAA GCAGGGGATT TTTATTGTAT TGAGGAAGAA GATGGGGTTT	480
GAGCCGTTGG ATTGGTATTG CAAGCCGGTG CCGAATGGTG TGTGGACTAA AACTGTGGAT	540
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CTGGTTCTGT TGATTCTGTG TCTTTATCGC TTGTGGCTCA TCACGAAGGA TCACAAAGTG	660
GATAAGTTCT GCTTGAGGTC TAAATGGTT AGCTATTTTC TGGCTCTTTT GGCTGCTTAT	720
GCTACTGCGG AGCCTTTGTT TAGATTGGTC ATGAGGATCT CTGTTTGGA TTTGGATGGA	780
GCTGGGTTTC CTCCCTATGA GGTGTGTTAT CACTTGCTG TTTTGTGAT GTTGTCTCC	840
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CTCTTTACAA AATGGTCGAA GGTAACGTTA TTCTTAAGAT TTCTGATACG AGTATACGAC	9780
ATAAAGAATT GTTGAAGTTT CTGATCTAA TAATTTGTGT ATATACTCTC AGGGCTTGCC	9840
GTGATGAGCA GATTGGCGAG GAACAGAATG CAACACCCGG ATTACAATTT AGAAGGGAAA	9900
TCGTTTGACT GGGACAATGT CGAGATGTAA ACGATGAAAG GCTTACACTA ATAGACCTAA	9960
AACTCCCATT TTGATGGAAC TTTTATTTGT ATTGCTTGGG ATACACGTAA CAAAATGCCC	10020
ATTAATCGTG GTGTAACAT ATAGGCTATG CTTCTTTTGG GAAAAAGAGA GTTTGATTAC	10080
AGAGGATGTG ATGATAACAC AATTGGAATT CAAATTTGCA GCAAAATTTG GGAGAAAAAA	10140
AAAAGTCAAT GAGTGCAACA TGCCAACATG GTTTCAACTT CTGGACATGG ACAACCATTG	10200
GACATAATTT CTCTCACAGG ACCATGTTTT GTCAATTGACA TTTTGCACAA AAATGTTCTA	10260
TTAAACATAT ATCTATAAAG AATTTGAACA ATTGTTAAAA AAACACTTAA AATATAAATT	10320

GCAATACAAA TTTCCTTTTT TT

10342

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1622 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Gly Phe Glu Pro Leu Asp Trp Tyr Cys Lys Pro Val Pro Asn Gly
 1           5           10           15

Val Trp Thr Lys Thr Val Asp Tyr Ala Phe Gly Ala Tyr Thr Pro Cys
      20           25           30

Ala Ile Asp Ser Phe Val Leu Gly Ile Ser His Leu Val Leu Leu Ile
      35           40           45

Leu Cys Leu Tyr Arg Leu Trp Leu Ile Thr Lys Asp His Lys Val Asp
      50           55           60

Lys Phe Cys Leu Arg Ser Lys Trp Phe Ser Tyr Phe Leu Ala Leu Leu
      65           70           75           80

Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Val Met Arg Ile
      85           90           95

Ser Val Leu Asp Leu Asp Gly Ala Gly Phe Pro Pro Tyr Glu Ala Phe
      100          105          110

Met Leu Val Leu Glu Ala Phe Ala Trp Gly Ser Ala Leu Val Met Thr
      115          120          125

Val Val Glu Thr Lys Thr Tyr Ile His Glu Leu Arg Trp Tyr Val Arg
      130          135          140

Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
      145          150          155          160

Val Leu Ser Val Lys Glu Tyr Tyr Gly Ser Phe Lys Leu Tyr Leu Tyr
      165          170          175

```


Ile Ser Glu Val Ala Val Gln Val Ala Phe Gly Thr Leu Leu Phe Val
 180 185 190
 Tyr Phe Pro Asn Leu Asp Pro Tyr Pro Gly Tyr Thr Pro Val Gly Thr
 195 200 205
 Glu Asn Ser Glu Asp Tyr Glu Tyr Glu Glu Leu Pro Gly Gly Glu Asn
 210 215 220
 Ile Cys Pro Glu Arg His Ala Asn Leu Phe Asp Ser Ile Phe Phe Ser
 225 230 235 240
 Trp Leu Asn Pro Leu Met Thr Leu Gly Ser Lys Arg Pro Leu Thr Glu
 245 250 255
 Lys Asp Val Trp His Leu Asp Thr Trp Asp Lys Thr Glu Thr Leu Met
 260 265 270
 Arg Ser Phe Gln Lys Ser Trp Asp Lys Glu Leu Glu Lys Pro Lys Pro
 275 280 285
 Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp
 290 295 300
 Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro
 305 310 315 320
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 325 330 335
 Trp Ile Gly Tyr Ile Tyr Ala Ile Ser Ile Phe Val Gly Val Val Leu
 340 345 350
 Gly Val Leu Cys Glu Ala Gln Tyr Phe Gln Asn Val Met Arg Val Gly
 355 360 365
 Tyr Arg Leu Arg Ser Ala Leu Ile Ala Ala Val Phe Arg Lys Ser Leu
 370 375 380
 Arg Leu Thr Asn Glu Gly Arg Lys Lys Phe Gln Thr Gly Lys Ile Thr
 385 390 395 400
 Asn Leu Met Thr Thr Asp Ala Glu Ser Leu Gln Gln Ile Cys Gln Ser
 405 410 415
 Leu His Thr Met Trp Ser Ala Pro Phe Arg Ile Ile Val Ala Leu Val
 420 425 430
 Leu Leu Tyr Gln Gln Leu Gly Val Ala Ser Ile Ile Gly Ala Leu Phe
 435 440 445

Leu Val Leu Met Phe Pro Ile Gln Thr Val Ile Ile Ser Lys Thr Gln
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Lys Leu Thr Lys Glu Gly Leu Gln Arg Thr Asp Lys Arg Ile Gly Leu
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 485 490 495

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Ser Trp Phe Arg Lys Ala Gln Leu Leu Ser Ala Phe Asn Met Phe Ile
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Leu Asn Ser Ile Pro Val Leu Val Thr Val Val Ser Phe Gly Val Phe
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Ser Leu Leu Gly Gly Asp Leu Thr Pro Ala Arg Ala Phe Thr Ser Leu
 545 550 555 560

Ser Leu Phe Ser Val Leu Arg Phe Pro Leu Phe Met Leu Pro Asn Ile
 565 570 575

Ile Thr Gln Met Val Asn Ala Asn Val Ser Leu Asn Arg Leu Glu Glu
 580 585 590

Val Leu Ser Thr Glu Glu Arg Val Leu Leu Pro Asn Pro Pro Ile Glu
 595 600 605

Pro Gly Gln Pro Ala Ile Ser Ile Arg Asn Gly Tyr Phe Ser Trp Asp
 610 615 620

Ser Lys Ala Asp Arg Pro Thr Leu Ser Asn Ile Asn Leu Asp Ile Pro
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Leu Gly Ser Leu Val Ala Val Val Gly Ser Thr Gly Glu Gly Lys Thr
 645 650 655

Ser Leu Ile Ser Ala Met Leu Gly Glu Leu Pro Ala Arg Ser Asp Ala
 660 665 670

Thr Val Thr Leu Arg Gly Ser Val Ala Tyr Val Pro Gln Val Ser Trp
 675 680 685

Ile Phe Asn Ala Thr Val Arg Asp Asn Ile Leu Phe Gly Ala Pro Phe
 690 695 700

Asp Gln Glu Lys Tyr Glu Arg Val Ile Asp Val Thr Ala Leu Gln His
 705 710 715 720

Asp Leu Glu Leu Leu Pro Gly Gly Asp Leu Thr Glu Ile Gly Glu Arg
 725 730 735
 Gly Val Asn Ile Ser Gly Gly Gln Lys Gln Arg Val Ser Met Ala Arg
 740 745 750
 Ala Val Tyr Ser Asn Ser Asp Val Cys Ile Leu Asp Glu Pro Leu Ser
 755 760 765
 Ala Leu Asp Ala His Val Gly Gln Gln Val Phe Glu Lys Cys Ile Lys
 770 775 780
 Arg Glu Leu Gly Gln Thr Thr Arg Val Leu Val Thr Asn Gln Leu His
 785 790 795 800
 Phe Leu Ser Gln Val Asp Lys Ile Leu Leu Val His Glu Gly Thr Val
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 835 840 845
 Asn Gly Glu Ala Glu Val His Gln Thr Ser Val Lys Pro Val Glu Asn
 850 855 860
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 865 870 875 880
 Lys Glu Gly Asn Ser Val Leu Val Lys Arg Glu Glu Arg Glu Thr Gly
 885 890 895
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 900 905 910
 Ala Trp Val Val Met Met Leu Val Ile Cys Tyr Val Leu Thr Gln Val
 915 920 925
 Phe Arg Val Ser Ser Ile Thr Trp Leu Ser Glu Trp Thr Asp Ser Gly
 930 935 940
 Thr Pro Lys Thr His Gly Pro Leu Phe Tyr Asn Ile Val Tyr Ala Leu
 945 950 955 960
 Leu Ser Phe Gly Gln Val Ser Val Thr Leu Ile Asn Ser Tyr Trp Leu
 965 970 975
 Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Met His Asp Ala Met Leu
 980 985 990

Gly Ser Ile Leu Arg Ala Pro Met Val Phe Phe Gln Thr Asn Pro Leu
 995 1000 1005
 Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Met Gly Asp Ile Asp Arg
 1010 1015 1020
 Thr Val Ala Val Phe Val Asn Met Phe Met Gly Ser Ile Ala Gln Leu
 1025 1030 1035 1040
 Leu Ser Thr Val Ile Leu Ile Gly Ile Val Ser Thr Leu Ser Leu Trp
 1045 1050 1055
 Ala Ile Met Pro Leu Leu Val Val Phe Tyr Gly Ala Tyr Leu Tyr Tyr
 1060 1065 1070
 Gln Asn Thr Ser Arg Glu Ile Lys Arg Met Asp Ser Thr Thr Arg Ser
 1075 1080 1085
 Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn Gly Leu Ser Ser Ile
 1090 1095 1100
 Arg Ala Tyr Lys Ala Tyr Asp Arg Met Ala Glu Ile Asn Gly Arg Ser
 1105 1110 1115 1120
 Met Asp Asn Asn Ile Arg Phe Thr Leu Val Asn Met Ala Ala Asn Arg
 1125 1130 1135
 Trp Leu Gly Ile Arg Leu Glu Val Leu Gly Gly Leu Met Val Trp Trp
 1140 1145 1150
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 1155 1160 1165
 Ala Tyr Ala Ser Thr Met Gly Leu Leu Leu Ser Tyr Ala Leu Ser Ile
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 Thr Ser Ser Leu Thr Ala Val Leu Arg Leu Ala Ser Leu Ala Glu Asn
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 Ser Leu Asn Ser Val Glu Arg Val Gly Asn Tyr Ile Glu Ile Pro Ser
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 Glu Ala Pro Leu Val Ile Glu Asn Asn Arg Pro Pro Pro Gly Trp Pro
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 Ser Ser Gly Ser Ile Lys Phe Glu Asp Val Val Leu Arg Tyr Arg Pro
 1235 1240 1245
 Glu Leu Pro Pro Val Leu His Gly Val Ser Phe Leu Ile Ser Pro Met
 1250 1255 1260

Asp Lys Val Gly Ile Val Gly Arg Thr Gly Ala Gly Lys Ser Ser Leu
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 Leu Asn Ala Leu Phe Arg Ile Val Glu Leu Glu Lys Gly Arg Ile Leu
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 Ile Asp Glu Cys Asp Ile Gly Arg Phe Gly Leu Met Asp Leu Arg Lys
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 Val Val Gly Ile Ile Pro Gln Ala Pro Val Leu Phe Ser Gly Thr Val
 1315 1320 1325
 Arg Phe Asn Leu Asp Pro Phe Ser Glu His Asn Asp Ala Asp Leu Trp
 1330 1335 1340
 Glu Ser Leu Glu Arg Ala His Leu Lys Asp Thr Ile Arg Arg Asn Pro
 1345 1350 1355 1360
 Leu Gly Leu Asp Ala Glu Val Thr Glu Ala Gly Glu Asn Phe Ser Val
 1365 1370 1375
 Gly Gln Arg Gln Leu Leu Ser Leu Ala Arg Ala Leu Leu Arg Arg Ser
 1380 1385 1390
 Lys Ile Leu Val Leu Asp Glu Ala Thr Ala Ala Val Asp Val Arg Thr
 1395 1400 1405
 Asp Val Leu Ile Gln Lys Thr Ile Arg Glu Glu Phe Lys Ser Cys Thr
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Glu Ile Glu Asp Asp Asn Ser Ile Leu Lys Lys Thr Lys Asp Ala Val
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 Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ile Glu
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 Leu Tyr Lys Met Val Glu Gly Leu Ala Val Met Ser Arg Leu Ala Arg
 1585 1590 1595 1600
 Asn Arg Met Gln His Pro Asp Tyr Asn Leu Glu Gly Lys Ser Phe Asp
 1605 1610 1615
 Trp Asp Asn Val Glu Met
 1620

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1251 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTCAC TTTTG TCCTTT TTTT CTTAAC ATCT ACTTTT GTCA TCAGCAA ATT ATCTGT AAAT 60
 AAGATAG GGT TTATG CTTAT TGCTACA ATG AACCTA ATCC TATGAT GTGT ATTGCA ATTT 120
 GCAACCAT GC GAGTTT AATT ATTTG TTTAC TGCTATA GTG ATCATTT TAT GATGTG TTTT 180
 TATTAATT AC AAAACAG AGC ATCAAAA ATC AAAAGA ACAT ATCGCA TAAAT CGAACT ATGC 240
 TAATACCT CT CCTCA ATCTT TGTTGT TGTT ATATTCA AGT AGCTTATT CT TTTGTT TTAT 300
 TTTACGAT TA GATTTC TCTA GAATTTA ATT TATATT ATTT AATCATA CT GATCAAG GTT 360
 TGTAGCTT AA TCAATAT CGT TATCGT GTCA TCCTGC AGAT TCAAAT GATC AAGTCTA ATA 420
 ATCTACTT AT ATGTATT ATA TATATT AGAT ACCACCA ACG AAACAAA ATC ATATTT CTAT 480
 AACATT TGTT TGGTTAA ATA TATTTA AGA TTTGTA ACAG TTGTT CGGGT TCAAA ACTAT 540

CACTTTGTAG TTGTAGGATG AGGAAAAGTC GTGATATGAT CATCTACTAA AATCATGTGT	600
TTTTTAAAGA ACATGATTTT CATTGGATAG TTTAATAAAT GTTAAAAAAA TACTAAGTGT	660
CAAAGAAGAG ATTTGAACCA TATGTAGAAT ACTTGATTCG AATTTTTCCT GACGAATAAT	720
CTAATATCCT TTTCTCAAAA GAAAAAATG TTTGTAACT TGGACACGAT ATTATTATCC	780
AACTTCCTTT CTAGATATTC ATTTTAAAT TACCTATATA TTTTATTTT CTCAAAATAT	840
ACTAAAAATT GGATAGAGCT ATTAAATAAA AAAGATAGAA TTTAGAGAGA AATAGCAACA	900
TAATGAATTA TAATATAAAT ATTTTGTAAG GAAATAACAA ACTTTATAGT TAGTTTGCCT	960
AATATAGAAA AAAGATACAG TTATTTACCC ATTTGTTTGT GTGTAAAAAA AGGAGTAAAA	1020
TAAACAGAGA AAAGAGCTTC TTGTTTTTAC TTGTGAACGT TATTGACTTT TCGGCCTCTC	1080
TCTCTTCTCT ATACAAATAT ATGGATCTTC ATTTCTTCGT ATAGTGTAAG CAGTGACGCA	1140
TCCATTTATC ATCATCTCCT TATAATCTC GAATCTGCCA CAGAGAGAGC GTGTGACAAA	1200
ATGAGTTCAT AAGATTCCGT TATCGTCTTC CTGATTCCTC CAAATCTCCG G	1251

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1368 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AAACAATTGG TGTATTTTGA ATTTTTCATG CAACGCACGT GAACAGCTTA ATTGCTTGAT	60
TGGAAACAAA CCTTTTLAGA ATTCATTAAT CAGTTTLAGG TGTTTTGGAA AATTAACGAA	120
CTATAGTGGA GATTAATTAA TTTTATATTA GTCTTTTSTA GTACACAAAT CGAAGTTTCC	180
TAGATTTTTT CAAAGTTGAA AATAATATTG ATAATATTTA TCAACAATGA ATCTACAAAA	240
ACATAATTTT TTTGCCAAAC AAATAACACC GAAACAAGAT TCATTCACTA TTTTGGTTT	300
AAAAAAAAAA ATCAAAATTA CACTATTATG AAGCCAATTT TTGTATGCAA AAAACCTGTA	360

TGTATCAATT TGTTTGTATT AAAAAGTAAG CATTTATGTC TTTTTTTTAT AAATAATAGA	420
AACACTTACT AGATGAATAG ATTTTTTGGT TTTAGAACAG AATACTATAA TTGTATTTAT	480
ATAGCTTTTT TATATTATTC GATATAGAAA AGTGTATATA TAGGAAAAAT GTACCATATA	540
CTGTCAATAA CATATTTGAT TCTAAATATA AATAGAATTG TTTTAAAGAA ATATGATCGT	600
TTATAATTAA ATGGTTTTTA ATGTCTTTTC TTGGGGCAAA AAACAAAGCT TGTCTTTCGT	660
CCATATATTT GCATCGTAAG GGGTGACGTA TCACTCTCTC TTTCTCTCAA ATATTATTCT	720
TCAATCTCTT TTTGGGGAAT CTTCGAGCAA ATTAGTGAGA GAACCCACCC ACTTCTTTC	780
TCATATGAGT ACATAAGATC CCTTTTGAGT TTTCGTGTTT TGCCAAAATC TCCAGGTAAA	840
GCTTCTCCCT TTTTCTCTGT TTTCTCTGTT TTGTTATTCT CCCTTTTCTC CATTGTAGCT	900
TTTCCTGTA AAGTGGGATT GATAGTTTTG TTTCATGGAT TTCAAATTG TGTTATTGA	960
CTCGATACCA TCTTAAATGC AGAGTCTTTT CGTGATAATA AAATTATGGA TTCGTTTCAA	1020
AGTTTTTTTT TTTTCGTATG GAAACACTT GAGCTCTCTC AATCTTGTAG TCTTGACTCT	1080
TGATGATTCT TCTATGTTCT CGTTGTGATT GCTTGTCACT GTTCTATCTT TATATATGAT	1140
TAAATGCAAT TTGCCCCCTT TTTACGCGCG AATGTATTTA TTATCTTTCG CACTCTGGGT	1200
CCATTTCTTG TCACTTGAGC ACATAATGAT TGATTATGA CTTTTTAAAG TTATGAAAAT	1260
TTATTATTTT TGTGCTATG GTTTTTTGA ATTAGAAGCT CATTTCAAAG TTGTTGATTT	1320
TCTTTCAGG GTAGGGAATT GGTGTGGTAG CTTGTGATGC ACTGTGTT	1368

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Cys Asp Glu Phe Gly

1

5

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Cys Asp His Ile Lys
1 5

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 12 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTGACAATAT GC

12

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 54 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTTCACAGT TTAAAGCGTA GTCTGGGACG TCGTATGGGT AATTTTCATT GACC

54

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAACTGCAGA TGGCTGGTAA TCTTGTTTC

29

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAA TTTTCATTGA

50

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGATTAGCC ATGCATGTCT

20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCTGGTACC AGACTTGCCC TCC

23

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAAAGTGGAT GTGGGACGGG C

21

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCCATATGTT TACTGGC

17

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAACCGGTGC GGCCGCCATG GGGTTTGAGC CGT

33

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AATTAACCCT CACTAAAGGG

20

What is claimed is:

1. An isolated DNA encoding a plant GS-X pump polypeptide.
2. The isolated DNA of claim 1, wherein said DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.
3. An isolated preparation of a polypeptide comprising a plant GS-X pump.
4. The isolated preparation of a polypeptide of claim 3, wherein said polypeptide is selected from the group consisting of *AtMRP1*, *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof having GS-X pump activity.
5. A recombinant cell comprising the isolated DNA of claim 1.
6. The recombinant cell of claim 5, wherein said cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.
7. A vector comprising the isolated DNA of claim 1.
8. An antibody specific for a plant GS-X pump polypeptide.
9. An isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.
10. A cell comprising the isolated preparation of a nucleic acid of claim 9.

11. A vector comprising the isolated preparation of a nucleic acid of claim 9.
12. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-X pump.
13. A transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.
14. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.
15. An isolated DNA comprising a plant GS-X pump promoter sequence.
16. The isolated DNA of claim 15, wherein said promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.
17. A cell comprising the isolated DNA of claim 15.
18. A vector comprising the isolated DNA of claim 15.
19. The isolated DNA of claim 15, further comprising a reporter gene operably fused thereto.
20. A transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising GS-X pump promoter sequence.

21. A method of identifying a compound capable of affecting the expression of a plant GS-X gene comprising

providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto,

adding to said cell a test compound, and

measuring the level of reporter gene activity in said cell, wherein a higher or a lower level of reporter gene activity in said cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that said test compound is capable of affecting the expression of a plant GS-X pump gene.

22. A method of removing xenobiotic toxins from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

23. A method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

24. A method of generating a transgenic pathogen resistant plant comprising introducing to the cells of said plant an isolated DNA encoding a GS-X pump, wherein said pump is capable of transporting glutathionated isoflavonoid alexins into the cells of said plant.

25. A method of manipulating plant pigmentation comprising modulating the expression of a GS-X pump protein in said plant, wherein said GS-X pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

26. A method of alleviating oxidative stress in a plant comprising introducing into the cells of said plant DNA encoding a GS-X pump, wherein said DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

27. A method of manipulating the expression of a gene in a plant cell comprising
operably fusing a GS-X pump promoter sequence to the DNA sequence encoding said gene to form a chimeric DNA, and
generating a transgenic plant, the cells of which comprise said chimeric DNA, wherein upon activation of said GS-X pump promoter sequence, the expression of said gene is manipulated.

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Fig. 1A

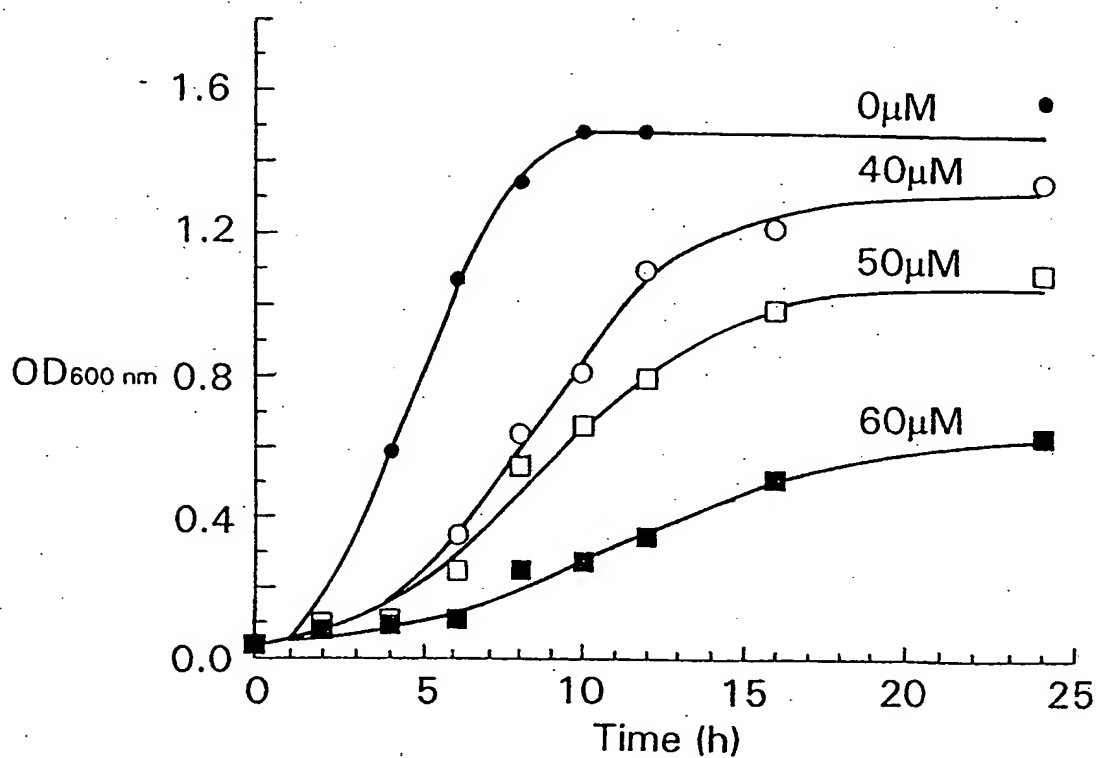
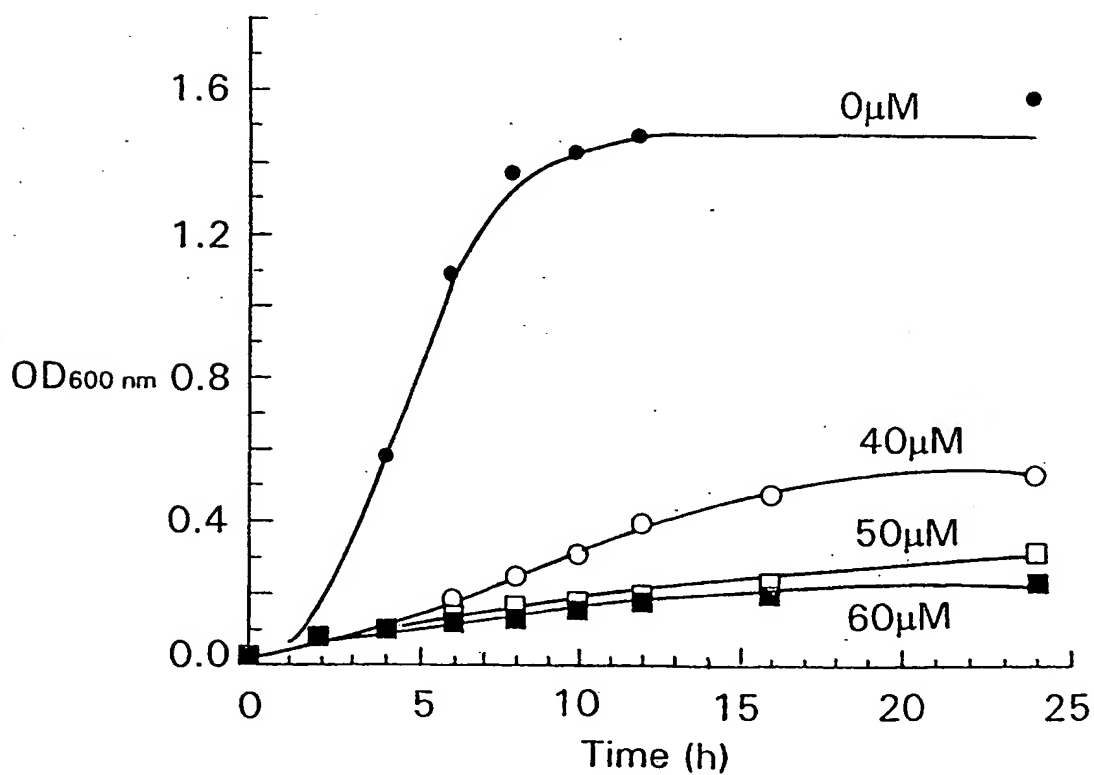
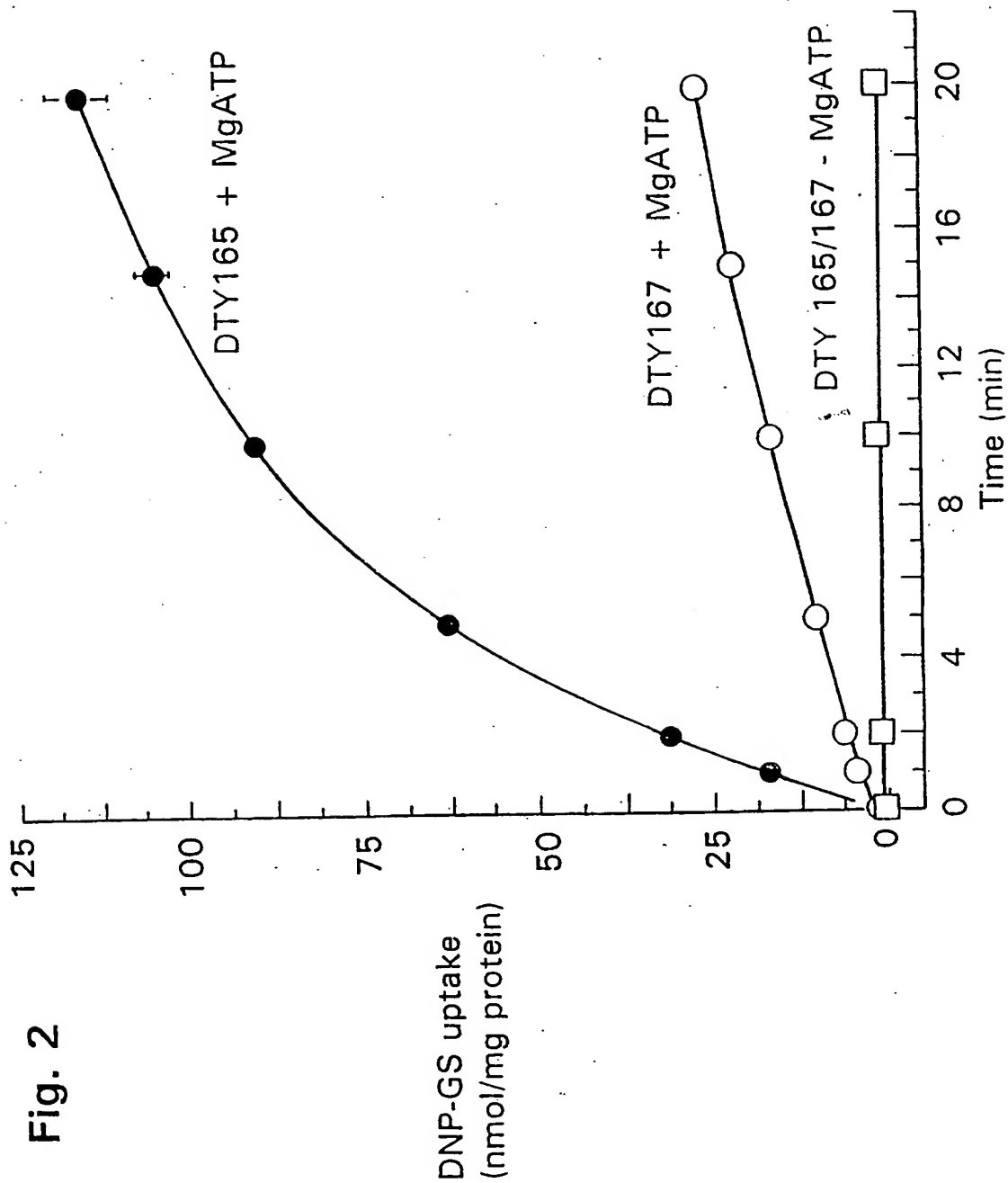


Fig. 1B



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Fig. 3A

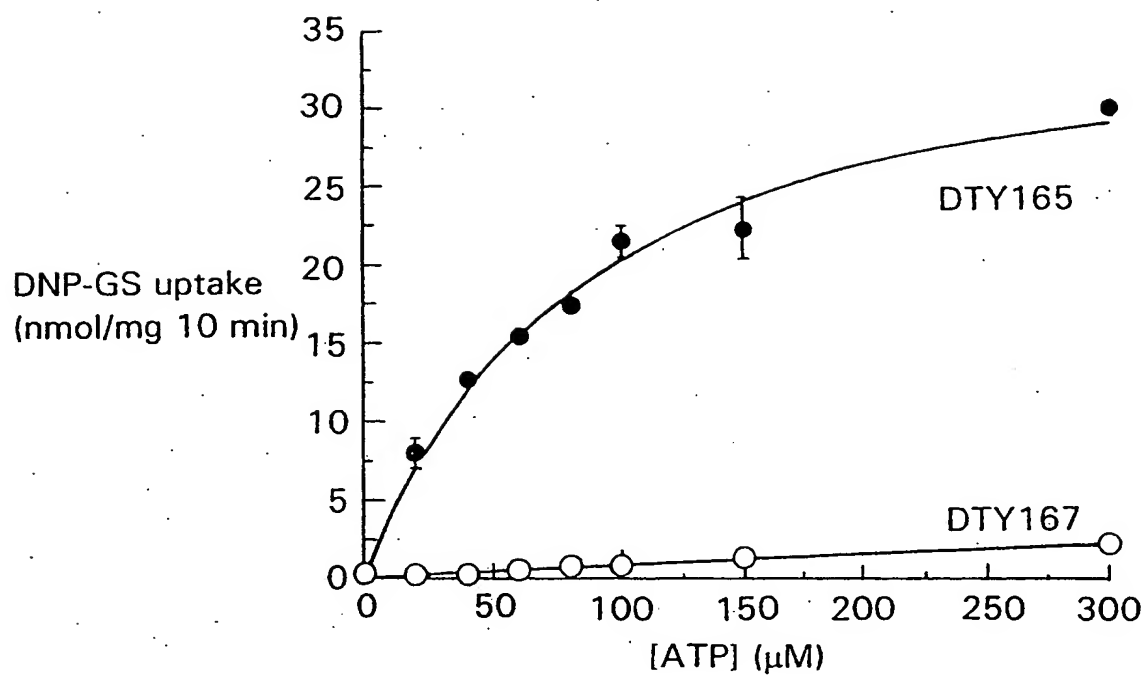
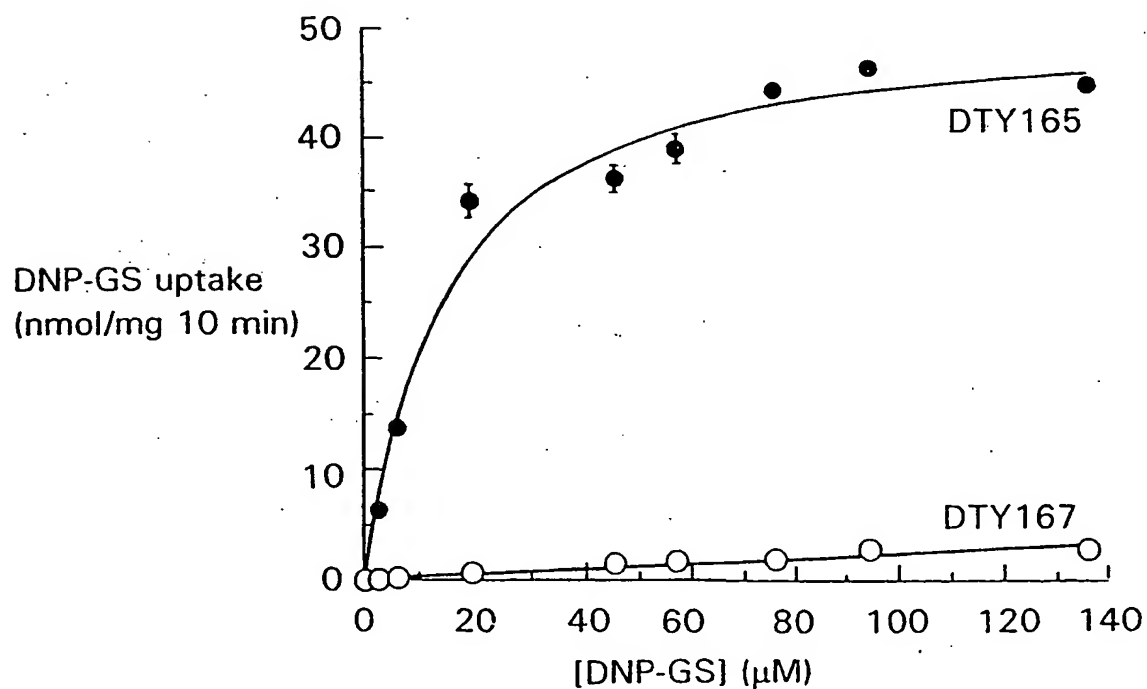


Fig. 3B



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Fig. 4A

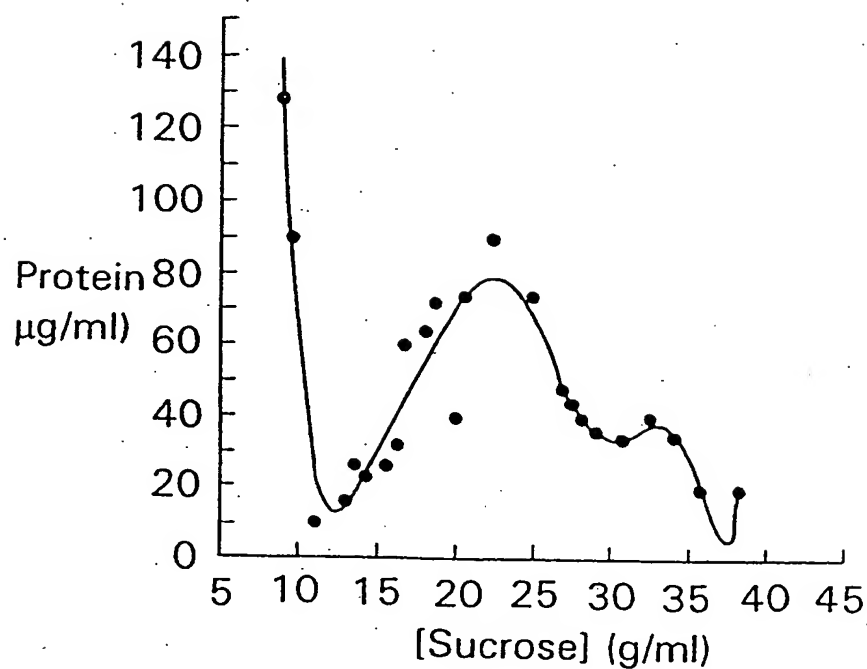
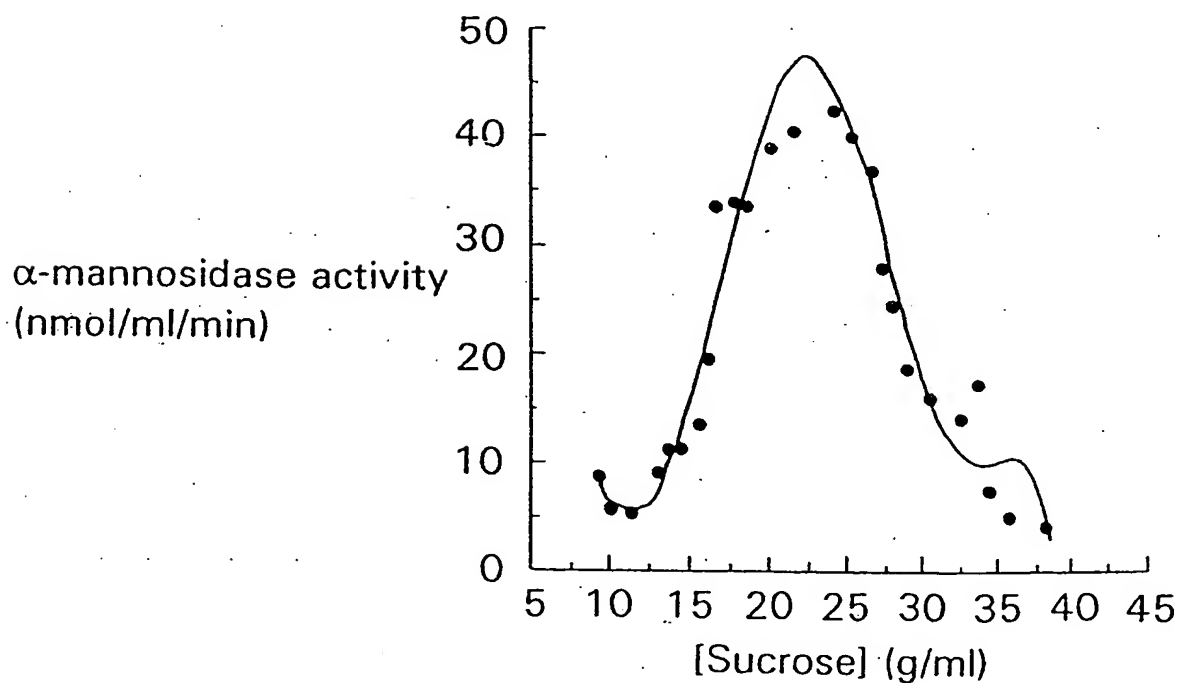


Fig. 4B



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Fig. 4C

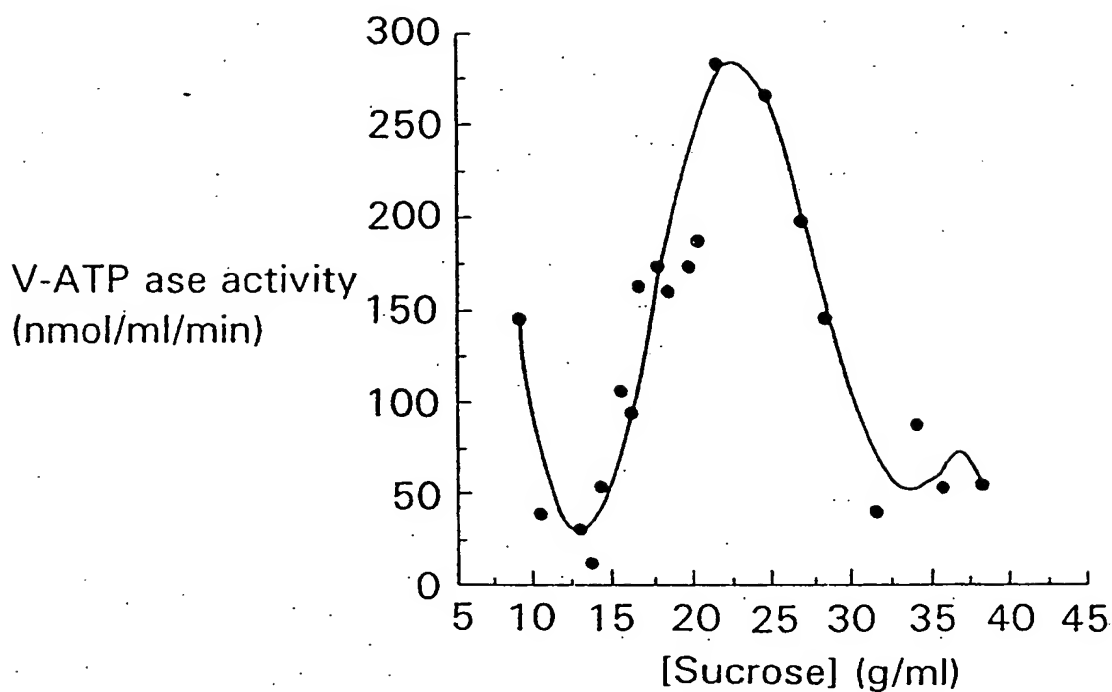
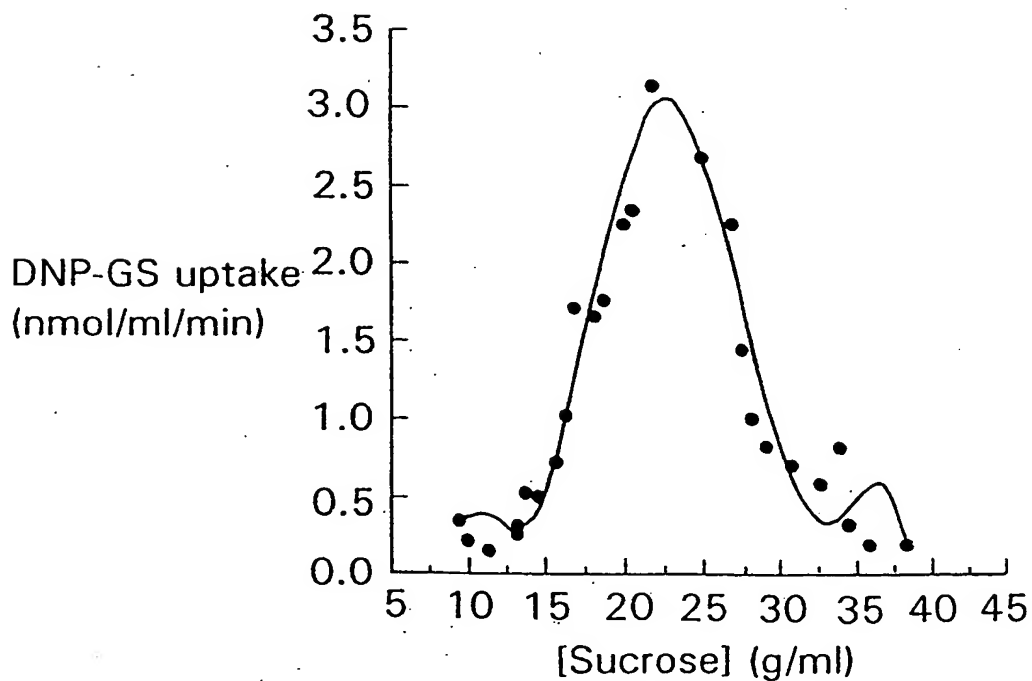


Fig. 4D



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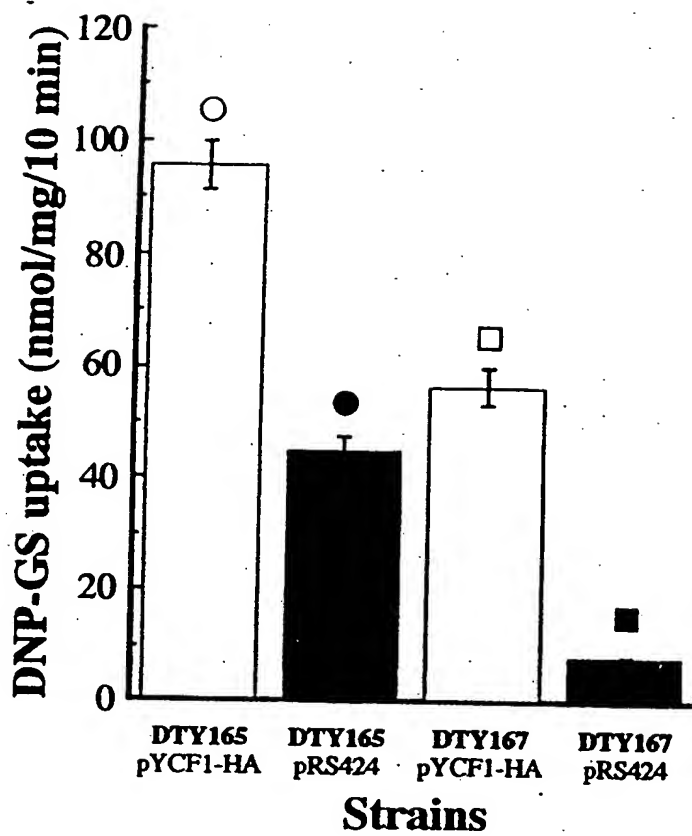


Fig. 5A.

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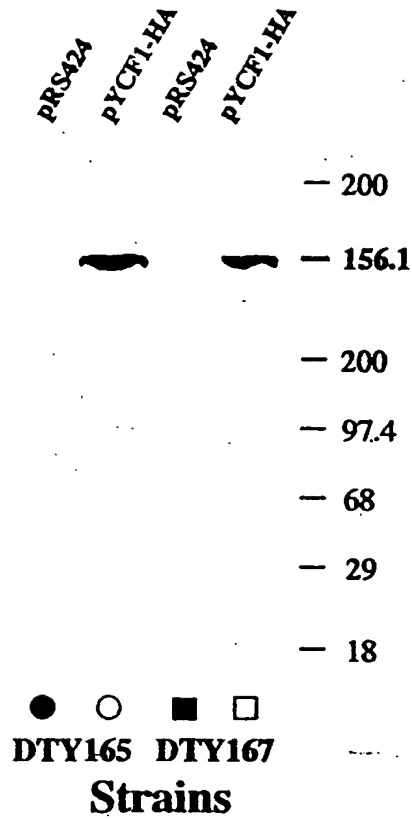


Fig. 5B

Fig. 6A

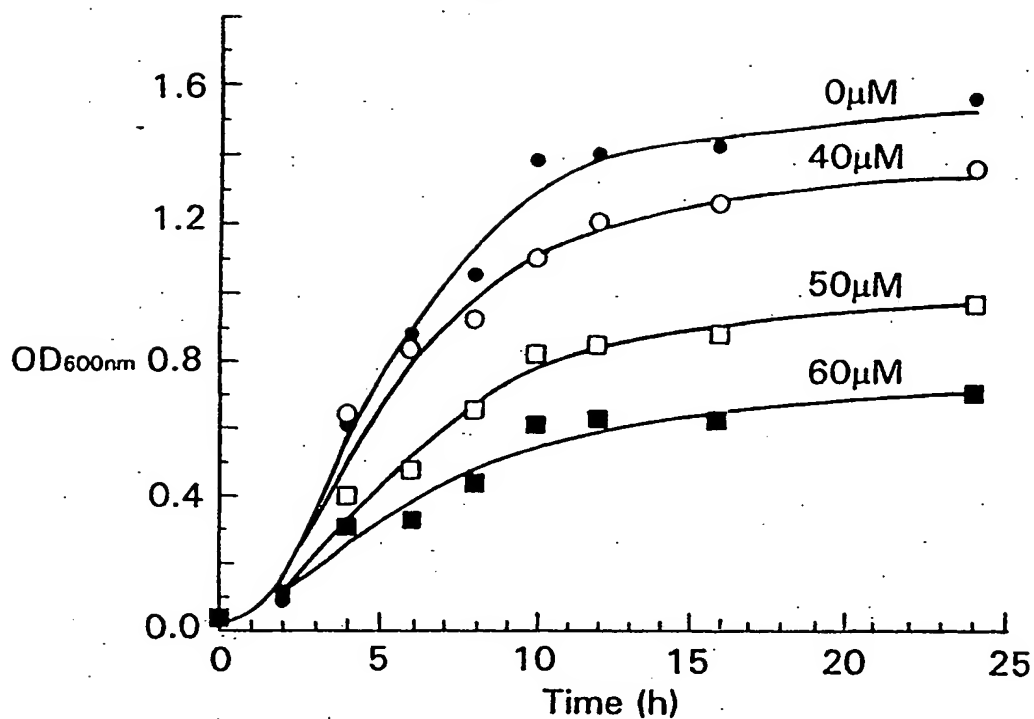
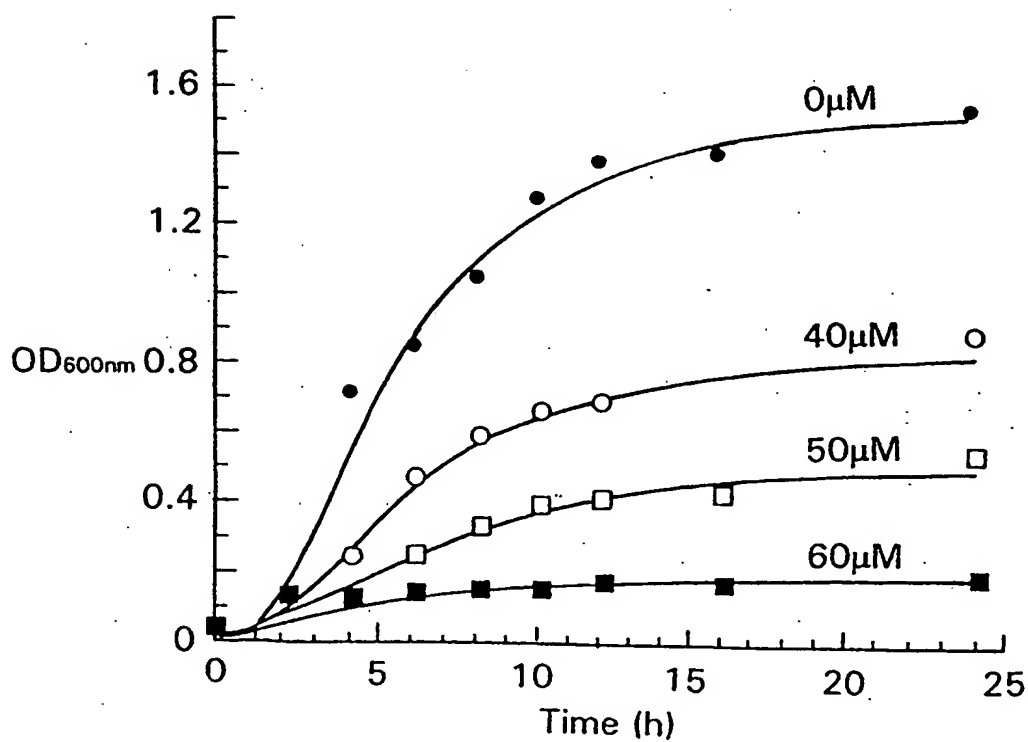


Fig. 6B



RECTIFIED SHEET (RULE 91)
ISA/EP

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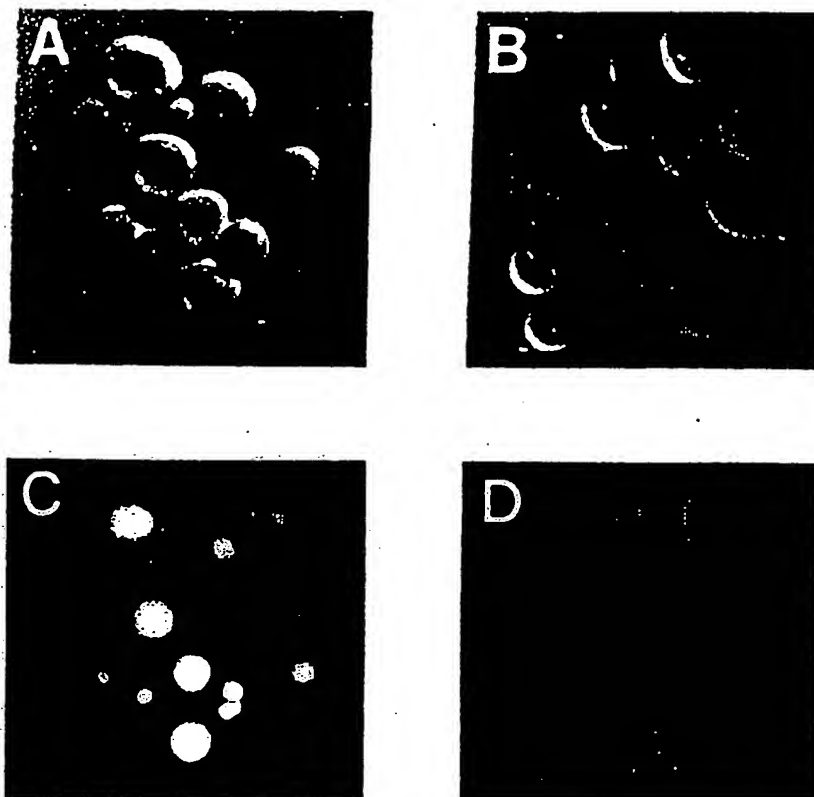


Fig. 7

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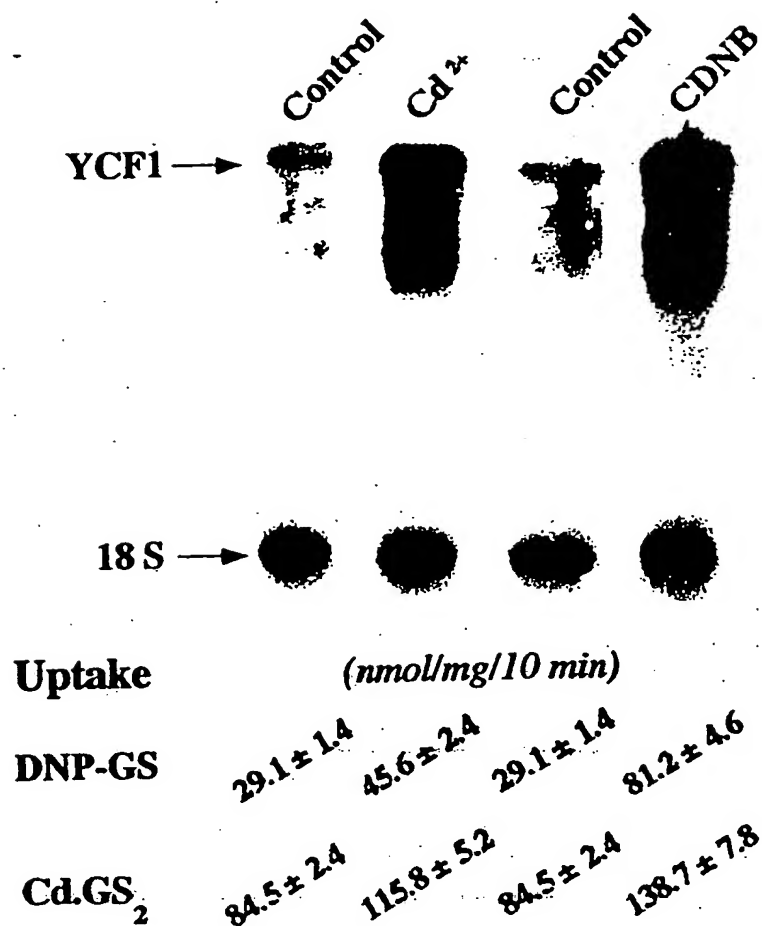


Fig. 12

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CTGACTCTTTTGTGCTTGGTATCTCTCAACTGGTTCTGTTGGTTCTGTGCCTGTATCGTA
TATGGCTCGCCTTAAAGGATCACAAGGTGGAGAGGTCTGTTTGGGTCGAGATTGTATA
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AAGTGACGATAAAAAATCCAAAGAAGGAAATAAAAAAGGAGGGAAATCTGTCTCATCAA
GCAAGAAGAACGTGAAACCGGAGTTGTAAGTTGGAGAGTCCTGAAGAGGTACCAGGATGC

FIGURE 13A

ACTTGGAGGGGCATGGGTAGTGATGATGCTCCTTTTATGTTACGTCTTAACAGAAGTATT
TCGGGTTACTAGCAGCACGTGGTTGAGTGAGTGGACTGATGCAGGAACCTCAAAGAGTCA
TGGACCCCTTTTCTACAATCTCATATATGCACTTCTCTCGTTTGGACAGGTTTGGTGAC
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CAATATGCTTCATTCCATACTGAGGGCCCCGATGTCCTTCTTCCATACCAATCCGCTAGG
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TGTTTATGCACAGTTTGGAGAGGCATTGAATGGCTTATCAACTATCCGTGCTTACAAAGC
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GACTCTGCGCAGTGTTCTCGAGGGGAAACACGACAAAGAGATTGCAGAGTCGCTTGAGGA
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ataaacttaattttcacctgcaaagaaaatcaaaccctgttggtgttcttcgtgataagta
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aaaaaaaaaaaaa

FIGURE 13B

gactcgataccatcttaaatgcagagtccttttcgtgataataaaaattatggattcgtttc
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FIGURE 14A

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TCCAAACATTATAACTCAGgtgatttcttaaatatgttggttgcaatgcatgtgtattaag
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TATGAAAGGGCCATTGATGTGACTTCACTGAAGCATGACCTAGAGTTACTGCCTGtaagt
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FIGURE 14B

aagtttgtttcaacctctttctcttgcttaatagGGTGGTGATCTCACGGAGATTGGAGA
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TCAACAGgtactaactcattgattctctttgataaggctagtctatttcatttttgaatt
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GTAATGGAAAATGCAGGGAAGGTGGAAGAATATTGAGAAGAAAATGGAGAAGCTGAGGCA
GACCAAAACAGCGGAACAACCAGTTGCGAATGGGAACACAAATGGTCTTCAAATGGATGGA
AGTGACGATAAAAAATCCAAAGAAGGAAATAAAAAAGGAGGGGAAATCTGTCCTCATCAAG
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GTATGGCTGATATCAACGGAAGATCAATGGATGATAATAACATCAGATTCACTCTGTCAACA
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CATCTGGATCCATAAAGTTTGGAGATGTTGTTCTCCGTTACCGCCCTCAGTTACCGCCTG
TGCTTCATGGGGTTTCTTTCTTCATTCCAAACAGATAAGGTGGGGATTGTTGGAAGGA
CTGGTGCTGGAAAGTCAAGCCTGTTGAATGCATTGTTTAGAATTGTGGAGGTGGAAAAAG
GAAGGATCTTAATCGATGATTGTGACGTTGGAAAGTTTGGACTGATGGACCTACGTAAG
TGCTCGGAATCATTCCACAGTCACCGGTTCTTTTCTCAGGAACTGTGAGGTTCAATCTTG
ATCCATTTGGTGAAACACAATGATGCTGATCTTTGGGAATCTCTAGAGAGGGCACACTTGA
AGGATACCATCCGCAGAAATCCTCTTGGTCTTGATGCTGAGgtattcagttgctgcctat
attgatatgaagtctcattttttaagtggtaataactgattttcaatctttgttcagGTC
TCTGAGGCAGGAGAGAATTTAGCGTGGGACAGAGGCAATTGTTGAGTCTTTCACGTGCG
CTGTTACGGAGATCTAAGATACTCGTCCTTGATGAAGCAACTGCTGCTGTAGATGTTAGA
ACCGATGCCCTCATTGAGAAGACTATCCGAGAAGAATTCAAGTCATGCACGATGCTCATT

FIGURE 14C

ATCGCTCACCGTCTCAATACCATCATTGACTGTGACAAAATTCTCGTGCTTGATTCTGGA
AGAgtagatgttttaaacactctctctctttcaatctcacactctccttggtttctcagcta
acctgttctattccaatttggttaactcagGTTCAAGAATTCAGTTCACCGGAGAACCTTC
TTTCAAATGAAGGAAGCTCTTTCTCCAAGATGGTTCAAAGCACTGGAGCTGCAAATGCTG
AGTACTTGCGTAGTTTAGTACTCGACAACAAGCGTGCCAAAGATGACTCACACCACTTAC
AAGGCCAAAGGAAATGGCTGGCTTCTTCTCGCTGGGCTGCAGCCGCTCAGTTTGCTCTGG
CTGCGAGTCTTACTTCGTGCGACAACGATCTTCAAAGCCTTGAAATTGAAGATGACAGCA
GCATTTTGAAGAGAACAAACGATGCAGTTGTGACTCTGCGCAGTGTTCTCGAGGGGAAAC
ACGACAAAGAGATTGCAGAGTCGCTTGAGGAACATAATATCTCTAGAGAGGGATGGTTGT
CATCACTCTATAGAATGGTAGAAGgtaaaccaaataatgcatctctacaaatgcttatgca
aaatcttaatcaccacactgaaacattaaagtcaaatcgtgctcttatattgcaagcctg
ctttccgctgtctacgtttcagGGCTTGCAGTGATGAGCAGATTGGCAAGGAACCGAATG
CAACAACCGGATTACAATTTCAAGGAAATACATTTGACTGGGACAACGTCGAGATGTAG
ATAAGTTCATGTTAAACTAGGAATCATTGTCTCTTCCGTAAGAAACATATATTTATCTTA
ACCAAATTTAGTTTGGTTTCCATTTTATAAACTTAATTTTACCTGCAAAGAAAATC
AAACCCTGTTGTGTTCTTCGTGATAAGTAGAGAAATTAATTGAGTATCCTTCTAACTCat
aatgggatctcatgattcatgaacaagcagcaacacaataataacccttttcagattttg
gagctggacaaagtgtgtaagttgagtttctcttacagtcattcatatacaaaaaacctct
tcgactgaagcaccaagaaagaaacaaacatcaaaagggaatgaggtcttttcttagggc
tgagatcatcggaatgtgggagtgcggaacacgacc

FIGURE 14D

SUBSTITUTE SHEET (RULE 26)

MGFEFIEWYCKPVPNGVWTKTVANAFGAYTPCATDSFVLGISQLVLLVLCYRIWLALKD
HKVERFCILRSRLYNYFLALLAAYATAEPLFRLIMGISVLDGPGPLPPFEAFGLGVKAFA
WGAVMVMILMETKIYIRELRWYVRFAYIYALVGDMVLLNLVLSVKEYYSSYVLYLYTSEV
GAQVLFGLLPMHLPNLDTPGYMPVRSETVDDYEYEEISDGQQICPEKHPNIFDKIFFS
WMNPLMTLGSKRPLTEKDVWYLDTWDTETLFTSFQHSWDKELQKPQPWLLRALNNSLGG
RFFWGGFWKIGNDCSQFVGPLLLNQLLKSMQEDAPAWMGYIYAFSIFGGVVFGVLCEAQY
FQNVMRVGYRLRSALIAAVFRKSLRLTNEGRRKFOTGKITNLMTTDAESLQQICQSLHTM
WSAPFRIIIALILLYQQLGVASLIGALLVLMFPLQTVIISKMQKLTKEGLQRTDKRIGL
MNEVLAAMDTVKCYAWENSFQSKVQTVRDELSWFRKSQLLGALNMFILNSIPVLVTIVS
FGVFTLLGGDLTPARAFTSLSLFAVLRFPFLMPLNIIITQVVNANVSLKRLEEVLATEERI
LLPNPPIEPGEPAISIRNGYFSWDSKGRPTLSNINLDVPLGSLVAVVGSTGEGKTSLSIS
AILGELPATSDAIVTLRGSVAYVPQVSWIFNATVRDNILFGSPFDREKYERAIDVTSKX
DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVYIFDDPLSALDAHVGQOVFE
KCIKRELQKTRVLVTNQLHFLSQVDRIVLVHEGTVKEEGTYEELSSNGPLFQRLMENAG
KVEEYSEENGEAEADQTAEQPVANGNTNGLQMDGSDDKKSKEGNKKGGKSVLIKQEERET
GVVSWRVLKRYQDALGGAWVVMMLLLCYVLTEVFRVTSSTWLSEWTDAGTPKSHGPLFYN
LIYALLSFGQVLVTLTNSYWLIMSSLYAAKKLHDNMLHSILRAPMSFFHTNPLGRIINRF
AKDLGDIDRTVAVFVNMFMGQVSQLLSTVVLIGIVSTLSLWAIMPLLVLFYGAYLYYQNT
AREVKRMDSISRSPVYAQFGEALNGLSTIRAYKAYDRMADINGRSMNINRFTLVNMGAN
RWLGIRLETGGMLMIWLTASFVAMQNGRAENQQAFASTMGLLLSYALNITSLLTGVLRLA
SLAENSLNAVERVGNIEIPPEAPPVIENNRPPPGWPSSSGSIKFEDVVLRYRPQLPPVLH
GVSFFIHPTDKVIGVGRTGAGKSSLLNALFRIVEVEEGRILIDDVDGKFGMLDLRKVLG
IIPQSPVLFSGTVRFNLDPFGEHNDADLWESLERAKLDTIRRNPLGLDAEVSEAGENFS
VGQRQLLSLSRALLRRSKILVLDEATAAVDVRTDALIQKTIREEFKSCTMLIIAHLNNTI
IDCDKILVLDGRVQEFSSPENLLSNEGSSFSKMVQSTGAANAAYLRSLVLDNKRKDDDS
HHLQGRKWASSRWAAAAQFALAASLTSSHNDLQSLEIEDDSSILKRTNDVAVTLRSVLE
GKHDKEAESLEEHNISREGWLSSLYRMVEGLAVMSRLARNRMQOPDYNFEGNTFDWDNVE
M

FIGURE 15

gaattcgcgccgcccgcgaatttgactctttacctctctttgactccgtgagattcga
ggattgttagtttcttgatgtgtagtctttgaagcaggggatttttattgtattgagg
aagaagATGGGGTTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGG
ACTAAAACTGTGGATTATGCGTTTGGTGCATACACGCCTTGTGCTATTGACTCTTTTGTG
CTTGGTATCTCTCATCTGGTTCTGTTGATTCTGTGTCTTTATCGCTTGTGGCTCATCACG
AAGGATCACAAAGTGGATAAGTTCTGCTTGGGTCTAAATGGTTTAGCTATTTTCTGGCT
CTTTTGGCTGCTTATGCTACTGCGGAGCCTTTGTTTAGATTGGTCATGAGGATCTCTGTT
TTGGATTGGATGGAGCTGGGTTTCTCCCTATGAGGCGTTTATGTTGGTCTTGAGGCT
TTTGCTTGGGGTTCTGCTTGGTTCATGACTGTTGTGGAACTAAAACGTATATCCATGAA
CTCCGTTGGTATGTCAGATTGCTGTCAATTATGCTCTTGTGGGAGACATGGTGTGTGTA
AATCTTGTCTCTCTGTTAAGGAGTACTATGGCAGTTTAAACTGTATCTTTACATAAGC
GAGGTGGCAGTTCAGGTTGCATTTGGAACCTCTTGTGTTGTGTATTTCCCTAATTTGGAC
CCTTACCTGGTTACACACCAGTTGGGACTGAAAATTCCGAGGATTACGAGTATGAAGAG
CTTCTGAGGAGAAAAATATATGTCCTGAGAGGCATGCAAAATTATTTGACAGTATCTTC
TTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGAT
GTATGGCATCTGGACACTTGGGATAAAACTGAACTCTTATGAGGAGCTTCCAGAAGTCC
TGGGATAAGGAACTAGAAAAGCCCAAACCGTGGCTTTTGGAGAGCACTGAACAACAGCCTT
GGGGGAAGGTTTGGTGGGGTGGCTTTTGAAGATTGGGAATGACTGTTTACAGTTCGTG
GGGCCTCTTCTACTGAATGAGCTCTTAAAGTCAATGCAACTTAATGAACCAGCGTGGATA
GGTTACATCTATGCAATCTCAATCTTTGTTGGAGTGGTATTGGGGGTTTATGTGAAGCT
CAGTATTTCCAAATGTGATGCGTGTGGTTACCGGCTTAGGTCTGCACTGATTGCTGCT
GTGTTCCGAAAATCTTTGAGGCTAACTAATGAGGGGCGGAAGAAGTTTCAAACAGGAAAA
ATAACAACTTAATGACTACTGATGCTGAGTCGCTGCAGCAAATCTGCCAATCACTTCAT
ACCATGTGGTCCGCGCCATTTCGTATAATTGTAGCACTGGTTCTCCTCTATCAACAATTG
GGTGTTCCTCGATCATTGGTGCATTGTTTCTGTCTCTTATGTTCCCATACAGACTGTT
ATTATAAGCAAAACGCAAGTTAACAAGAAAGGGTTGCAGCGTACTGACAAGAGAATT
GGCCTAATGAATGAGGTTTAGCGGCAATGGATACAGTGAAGTGTACGTTGGGAAAAC
AGTTTTTCAAGGTTCAAAGCTGTACGTGATGATGAATTATCTTGGTTCCGGAAAGCA
CAACTCCTGTCAGCGTTCAATATGTTTCACTAAACAGCATCCCTGTCCTCGTCACTGTT
GTTTCATTGTTGTTCTCATGCTTGGAGGAGATCTGACACCTGCAAGAGCGTTTACG
TCACTCTCTATTTTCTGTGCTTCGCTTCCCTTTATTATGCTTCCAAACATTATAACT
CAGATGGTAAATGCTAATGTATCCTAAACCGTTTGGAGGAGGTACTGTCAACCGAAGAGA
GAGTTCTCTTACCGAATCTCCCATTTGAACCTGGACAGCCAGCTATCTCAATAAGAAATG
GATACTTCTCCTGGGATTCAAAGGCGGATAGGCCAACATTGTCAAACATCAACCTGGACA
TACCTCTTGGCAGCCTAGTTGCGGTAGTTGGCAGCACAGGAGAAGGAAAAACCTCCCTGA
TATCTGCTATGCTTGGGGAACCTCCTGCAAGATCTGATGCGACTGTTACTCTTAGAGGAT
CAGTCGCTTATGTTCCACAAGTTTCATGGATCTTTAACGCAACAGTACGTGACAATATAT
TGTTTGGGGCTCCTTTTGAACCAAGAAAAATATGAAAGGGTGATTGATGTGACAGCACTCC
AGCATGACCTTGAGTTACTGCCTGGAGGTGACCTCACGGAGATCGGAGAAAGGGGTGTTA
ACATCAGTGGGGGACAAAAGCAGAGGGTTTCTATGGCTAGGGCCGTTTACTCAAATTCAG
ACGTGTGCATCTTAGATGAACCATTGAGTGCCCTTGATGCGCATGTTGGTCAAGAGTTT
TTGAAAAATGCATAAAAAGGGAACCTAGGGCAGACAACGAGAGTACTTGTTACAAATCAGC
TCCACTTCTATCACAAGTGGATAAAATCTACTTGTCCATGAGGGAACAGTAAAAGAGG
AAGGAACATATGAAGAATTATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAATG
CAGGGAAGGTTGAAGATTATCCGAAGAAAATGGAGAAGCTGAAGTACATCAAACATCTG
TAAAACCAAGTTGAAAATGGGAACGCTAATAATCTGCAGAAGGATGGAATCGAGACAAAG
ATTCCAAAGAAGGAACTCTGTTCTTGTCAAACGAGAAGAACGTGAAACTGGAGTTGTGA
GTTGGAAAGTCTGGAGAGGTACCAGAATGCACTTGGAGGTGCATGGGTAGTGATGATGC

FIGURE 16A

TCGTTATATGCTACGTCTTGACTCAAGTATTTCCGGGTTTCAAGCATCACTTGGTTGAGTG
AGTGGACTGATTCAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATG
CGCTTCTTTTCGTTTGGACAGGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGT
CCAGTCTATATGCAGCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTC
CAATGGTGTCTTTCAAACCAATCCATTAGGACGGATAATCAATCGATTTGCAAAAGATA
TGGGAGATATTGATCGAACTGTGGCAGTCTTTGTAAACATGTTTATGGGTTCAATCGCAC
AGCTTCTTTCAACTGTTATCTTGATTGGCATTGTGAGCACTCTGTCCCTGTGGGCCATCA
TGCCCCCTGTTGGTCGTGTTCTATGGAGCTTATCTGTATTACCAGAACACATCTCGGGAAA
TTAAACGTATGGATTCCACTACAAGATCGCCAGTTTATGCTCAATTTGGTGAGGCATTGA
ATGGACTATCTAGTATCCGTGCTTATAAAGCATATGACAGGATGGCTGAAATTAATGGAA
GGTCAATGGACAATAACATCAGATTCACTTGTAAACATGGCTGCAAATCGGTGGCTGG
GAATCCGTTTGGAAAGTTTGGGAGGTCTCATGGTTTGGTGGAAGTCTTCAATAGCCGTCA
TGCAGAACGGAAAGGCAGCGAACCAACAAGCATATGCATCTACGATGGGTTTGCCTTCTCA
GTTATGCGTTAAGCATTACCAGCTCTTTAACAGCTGTACTGAGACTCGCGAGTCTAGCTG
AGAATAGTTTAAACTCGGTTGAGCGTGTGGAAATTATATCGAGATACCATCAGAGGCTC
CATTGGTCATTGAAAACAACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAAT
TTGAGGATGTTGTTCTTCGTTACCGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGT
TCTTGATTTCTCCAATGGATAAGGTGGGAATTGTTGGGAGGACAGGCGCTGGGAAATCAA
GCCTCTTAAATGCCTTATTTCAGGATTGTGGAGCTGGAAAAAGGAAGGATTTTAATTGATG
AATGCGACATTGGAAGATTTGGACTGATGGACCTACGTAAAGTGGTCGGAATTATACCGC
AAGCGCCAGTTCTTTTCTCAGGTACCGTGAGATTCAATCTTGACCCATTTAGTGAACACA
ACGACGCCGATCTCTGGGAATCTCTTGAGAGGGCACACTTGAAAGATACTATCCGCAGAA
ATCCTCTTGGTCTTGATGCTGAGGTAAGTGGGAGGAGAGAAATTCAGTGTGGACAGA
GACAGTTGTTGAGTCTTGACCGTGCAATTGTTACGAAGATCTAAGATACTTGTCTTGATG
AAGCAACTGCTGCAGTTGACGTAAGAAGTATGTTCTCATCCAAAAGACCATCCGAGAAG
AATTCAAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTG
ACAAAGTTCTTGTCCTTGATTCTGGAAAAGTTCAGGAATTCAGTTCACCGGAGAATCTTC
TTTCAAATGGAGAAAGTTCTTCTCGAAGATGGTTCAAAGTACAGGAAGTGCAAACGCGG
AGTACTTACGTAGTATAACACTAGAGAACAACGTAACAGAGAAGCTAACGGTGATGATT
CACAACCTTTAGAAGGTCAAAGGAAATGGCAAGCTTCTTCTCGTTGGGCTGCAGCTGCTC
AATTTGCATTGGCTGTGAGCCCTCACTTCATCTCACAACGACCTCCAAAGCCTTGAAATCG
AAGATGATAACAGTATTTTGAAGAAAACAAGGACGCGCTCGTCACTTTACGCAGTGTCC
TTGAAGGGAAACATGATAAAGAGATTGAAGACTCTCTAAACCAAAGTGACATCTCTAGAG
AGCGTTGGTGGCCATCTCTTACAAAATGGTTCGAAGGGCTTGCCGTGATGAGCAGATTGG
CGAGGAACAGAAATGCAACACCCGGATTACAATTTAGAAGGGAAATCGTTTGACTGGGACA
ATGTCGAGATGTAAacgatgaaaggcttacactaatagacctaaaactcccatthtgatg
gaactthttatthtgattgcttgggatacacgtaacaaaatgccattaatcgtgggtgtaa
ctatataggctatgcttctthttgggaaaaagagagtttgattacagaggatgtgatgata
acacaattggaattc

FIGURE 16B

gggagggttgggtttttccctatcaatcgaattccatttcgtgctcgtaacgtggattttggtaga
tttttttagggggatggaaacttggttattatctatagatgatgattttgttttctccatgagaa
tgtatgcttttaaacttttttttttggtttttgccttcggagctaactttgggggctggtctcg
gtctctgttttctctccactaaaaagataaaaagcttttgccatcttttttttctcaataatc
tatcacatcggtttttttctttgttttttctccatttgcttctcattgagttcatagccacataat
tattgatttctttttcttttagtggttctgttactgatgcgtttcattatttatacttctcacttg
cagattcgaggattggttagtttcttgtagtgtagtcttgaagcaggggatttttattgtattg
aggaagaagATGGGGTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGGACT
AAAACGTGTGGATTATGCGTTTGGTGCATACACGCCCTTGTGCTATTGACTCTTTTGTGCTTGGTATC
TCTCATCTGGTTCTGTTGATTCTGTGTCTTTATCGCTTGTGGCTCATCACGAAGGATCACAAAGTG
GATAAGTCTGCTTGAGGTCTAAATGGTTTAGCTATTTTCTGGCTCTTTTGGCTGCTTATGCTACT
GCGGAGCCTTTGTTTAGATTGGTCATGAGGATCTCTGTTTGGATTGGATGGAGCTGGGTTTCCT
CCCTATGAGgtgtgttatcactttgctgttttggtgatgtgttctccttctgtatgtttttcct
gagagatgctgttgttttggtgttttattggcagGCGTTTATGTTGGTCCTTGAGGCTTTTGCTTG
GGGTTCTGCTTTGGTCATGACTGTTGTGGAACTAAAACGTATATCCATGAACTCCGTTGGTATGT
CAGATTCGCTGTCATTTATGCTCTTGTGGGAGACATGGTGTGTAAATCTTGTCTCTCTGTTAA
GGAGTACTATGGCAGgttggtaaatttgcaagtctgtatggtttatgcaattttgtttccctgggtct
ggcagatgaacttatatgcgtcatttttttttggtttttggcagTTTTAACTGTATCTTTACAT
AAGCGAGGTGGCAGTTCAGgtttgcactttaaaactccttttgcatctccaaactactctttac
catgtgctgtatctaagtcacactgtaaatgatacaactttgtttttataatgacgttaaggatgg
tttttggatccagGTTGCATTTGGAACCCCTCTGTTTGTGTATTCCCTAATTGGACCCTTACCC
TGTTTACACACCAGTTGGGACTGAAAATTCCGAGGATTACGAGTATGAAGAGCTTCTGGAGGAGA
AAATATATGTCCTGAGAGGEATGCAAATTTATTTGACAgtagtgcactctacacttctcattccct
actttgtttttataggtgcattttctattttaattgtgagaattgccaccgcacattttatcactt
ttctgcacttactacatctaagttggttatttatgcagagcttaaatatttccctggaattgtga
aattttcttatggagtgttaatacgtagtaggtcattaaaattgtttccgcagagagtagtctata
gtctcttcaaaatttttttgacttatcctccgttctccctagaaatgaacttatgatttgtga
ctgtgccgagggtttttgcttagtgatcatcacttcgactaagctgcaacattttatatagtatatt
cgtcaacattttgtcaaaactttgactattatgttcccttcttacccttgtctttcaaccacagGTAT
CTTCTTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGATGT
ATGGCATCTGGACACTTGGGATAAAACTGAACTCTTATGAGGAGgtatatatttaataaataacaa
ctgttctcactgtctatgactggcatggttgcgtagacatatttttatctcatttttttagCTTCC
AGAAGTCTGGGATAAGGAACTAGAAAAGCCCAAACCGTGGCTTTTGAGAGCACTGAACAACAGCC
TTGGGGGAAGgttaacaaaaaacttcttcacagtcagtgttttcatctttttgggctttgacatga
tgtgtgatttgtaaaaggaagcatttggttgtaataataaatgcattatgaataactagaagctga
gaaatctgttatggctgtgacttcaagtagtgttttgatgcgtgtcgagttgaataagaaatgtgtt
acttttctggttataatctgccatagatacttccatccttatggactgtctgtttctgcattttg
tagGTTTGGTGGGGTGGCTTTTGAAGgtacttttgtagtctttattgtgttttattctttatct
tgaaacagtcttttcccttgctctatttgataaatattgatggcttctgaggtcttagtttccctaaat
gggtgtgttttgtaactgtttaatcttgacatttcaatctaaattgtatcatagATTGGGAATGACT
GTTTACAGTTTCGTGGGGCCTCTTCTACTGAATGAGCTCTTAAAGgtttgttcccttacttctttt
accccgtagcatttggtgttgaaactatttaacacaatgctttgtaattttccattccatggat
ctttgagatggattcatattcctactggctcgaataagtggttaaacgttcttgatagattcaaaa
tccatcatcctttgaatattatgttctgacgatattccacaatgtctcctttaacttccgcagT
CAATGCAACTTAATGAACCAGCGTGGATAGGTTACATCTATGCAATCTCAATCTTTGTTGGAGTg
tatgcaacaaattctcttttctctcgctgcctttattattctcttgcatggactgcaaggatag
aaacaaaaactctacttctcttggttcttttcttcttgctaggacttcatgggtatttttgggtct
agagtagatgctacgaattgtaggaccagtttaattttcttaagctgaaagtaattctctgtgcgat
tcgattgtattagaaaatagcctgattctactcttagagttagttttttttgtttgttaatacatt
tgcatgttgaaaagggttttggttaattgtaggtcaagggtgacacttgaccaatggactccttgatcg
cttgatgttgatgttgacattttcagGTATTGGGGGTTTATGTGAAGCTCAGTATTTCCAAAATG
TGATGCGTGTGGTTACCGGCTTAGGTCTGCACTGgtaagaaaaagtttcacatgaattatctttt
gctacttagtttttctt

FIGURE 17A

SUBSTITUTE SHEET (RULE 26)

tttgctctgcttctcatggttttgatgcaatacctgtactgttatgtctgttgaaagctatagcaga
tgcttatagattgcttcattctgctgatgaattctcccttaataagATTGCTGCTGTGTTCCGAAAA
TCTTTGAGGCTAACTAATGAGGGGCGGAAGAAGTTTCAAACAGGAAAAATAACAACTTAATGACT
ACTGATGCTGAGTCGCTGCAGgtgtatctttgttacctttactctctttagccttgtctgtttctt
gatataaatttacactgcatagttgtatatctacctcaaaatatgagtccttagatgcaatttacca
agatagtccttttctgcaactgacgactgaatctgaagcttattctaagattctagaaatcctaa
gagttgtgattacattttcaacacccttgttcttttgttgccgttgtaggatttgattttccttta
ttagccaataaacctttaattcgcttgatttgtagaaaaaagttacctttgaacagtgcttttctc
taagctcttgcttgaaatcaaagtggttatctagctgatagctgttcttttccctaacgtttctc
ttgtgtgtgacagCAAATCTGCCAATCACTTCATACCATGTGGTTCGGCGCCATTTCTGTATAATTGT
AGCACTGGTTCCTCTATCAACAATTGGGTGTTGCCTCGATCATTGGTGCATTGTTTCTGTCTCT
TATGTTCCCATACAGgttcgtatatcttaataattccccattctctttgctgtctcggttttttt
ttccttttgattgcttatttctcatttgcttttcacaccaatgaaaatgattcatttctcctcggtt
atttgggtgaaacagACTGTTATTATAAGCAAAACGCAGAAAGTTAAACAAAAGAAGGGTTGCAGCGT
ACTGACAAGAGAATTGGCCTAATGAATGAGGTTTACGCGCAATGGATACAGTGAAGtacgatact
ttggaagcctgaaacctaataatttatttcttgcatagttgggaagtttgtggcagtggttaactat
ctcactaaaccaaataactgtagGTGTTACGCTTGGGAAAAACAGTTTTTCAGTCCAAGGTTCAAAC
GTACGTGATGATGAATTATCTTGGTTCGGGAAAGCACAACTCCTGTGTCAGCGgtatggcttgagtg
agtgactgttatattaattgattttatagaccgtatgcatgatgtgcatagttgtcttgggtcattt
acttgtcgctctcctaaccggtatgattgtatacaaggacaaatccaagttgctcgtctttttaaat
gcctttgaccattttgagaatggtatccatcaatatgtgtttaggcattttctgtactattttcta
gttcattgaacattgattcagttgtttcgggcatgtgtagcagcattcatgcatgatctttaacat
atattgcattaatgtttctgactcattcttgggtcttctatttgcctctgcagTTCAATATGTTTATA
CTAAACAGCATCCCTGTCTCTCGTGACTGTTGTTTTCATTGGTGTGTTCTCATTGCTTGGAGGAGAT
CTGACACCTGCAAGAGCGTTTACGTCACTCTCTCTATTTTCTGTGCTTCGCTTCCCTTTATTTCATG
CTTCCAAACATTATAACTCAGgtgatttctttaaagtgttcttgaaccatgttttcatgtccagt
actgaataatgtggcatcatagtaatgattgcttctgattgctcttttaattttccatctctacct
cttttctagaccagtcgttgtcataatgttttgcagatgctgaccaggcttcttctttagatAT
GGTAAATGCTAATGTATCCTTAAACCGTTTGGAGGAGGTAAGTCAACCGAAGAGAGAGTTCTCTT
ACCGAATCCTCCCATGTAACCTGGACAGCCAGCTATCTCAATAAGAAATGGATACTTCTCCTGGGA
TTCAAAGgtcttcttcttcttatttcatcacatgttcttacttctattagtttctatcattacatat
tgtcaatgaagtacaaaaagtgagctagaagtatacatatgcagGCGGATAGGCCAACATTGTCAA
ACATCAACCTGGACATACCTCTTGGCAGCCTAGTTGCGGTAGTTGGCAGCACAGGAGAAGGAAAA
CCTCCCTGATATCTGCTATGCTTGGGGAACCTCCTGCAAGATCTGATGCGACTGTTACTCTTAGAG
GATCAGTGCCTTATGTTCCACAAGTTTCATGGATCTTTAACGCAACagtaagtttatatatgctac
tcagtttatagtatggttctcaatgcgaaaatgtcaaatctcctcttggattgttacttattttg
tatgtattttatgttttgtatatgatgatgtgtgcttttagatacgtccacatgctgatggttgta
attaacatcgcttagGTACGTGACAATATATTGTTTGGGGCTCCTTTTGACCAAGAAAAATATGAA
AGGGTGATTGATGTGACAGCACTCCAGCATGACCTTGAGTTACTGCCTgtaagttttgtggagagt
tacttagccatgtgcattgaaaatttcttgaggtgaaacgaacctgaaatctgttggtgcatgt
aaatcgaaaaaactgaattgcatcagttctgttgatagcatgtacttctattttctagtgtcagg
tatctaagcttgttttcttcttcttcttcttgattgatagGGAGGTGACCTCACGGAGATCGGAGAA
AGGGGTGTTAACATCAGTGGGGGACAAAAGCAGAGGGTTTCTATGGCTAGGGCCGTTTACTCAAAT
TCAGACGTGTGCATCTTAGATGAACCATTGAGTGCCCTTGATGCGCATGTTGGTTCAGCAGgtaa
tagccataggctcttttggatagaacaatactttgttttcttcttcaattttgcaaatcgtgaactc
tataacgttttgttttcaatctgcatggatattctacttcttgtttgccacggatctctgccata
tactacttttaagcaaacattgttatctgatgttcgaaactggctgttatatatagGTTTTTGAAA
AATGCATAAAAAGGGAACTAGGGCAGACAACGAGAGTACTTGTTACAAATCAGCTCCACTTCCTAT
CACAAGTGGATAAAAATCCTACTTGTCCATGAGGGAACAGTAAAAGAGGAAGGAACATATGAAGAAT
TATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAAATGCAGGGAAGGTTGAAGATTATCCG
AAGAAAAATGGAGAAGCTGAAGTACATCAAACATCTGTAAAACAGTTGAAAATGGGAACGCTAATA
ATCTGCAGAAGGATGGAATCG

FIGURE 17B
SUBSTITUTE SHEET (RULE 26)

AGACAAAGAATTCCAAAGAAGGAAACTCTGTTCTTGTCAAACGAGAAGAACGTGAAACTGGAGTTG
TGAGTTGGAAAGTCCTGGAGAGGtaagttggcattcggatttttgctctttcttggttggttggtg
cagtatctctttctatcgacagtggaaatatccgtaaataagacatattctttgggttagagcaat
atgtcaatttatctgtggtggtttctttactacaaaatggatatatattggttgactcgctctattc
atattcatacaaaatgtatatatatattttccgtattaagggttcgtattgtaaagccattgtaataac
ttgtgaggtgtcaccatgttccagGTACCAGAAATGCACTTGGAGGTGCATGGGTAGTGATGATGCT
CGTTATATGCTACGTCTTGACTCAAGTATTTCCGGTTTCAAGCATCACTTGGTTGAGTGAGTGGAC
TGATTACAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATGCGCTTCTTTCGTT
TGGACAGgtatgagttgcatttggcaaatgtttgagtcggtatcttcatgatcggataacaatata
taactgaacattaaaggctgatcagttagaataacacacatgtttcttctgcgcaaaagtatcga
gcaaacaaaatggaaaataaaaggatacagagagcaaaacgtttatttgctaacacgtatttctgcg
ggggtttgtcagGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGTCCAGTCTATATGCA
GCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTCCAATGGTGTTCTTTCAAACC
AATCCATTAGGACGGATAATCAATCGATTTGCAAAAGATATGGGAGATATTGATCGAACTGTGGCA
GCTTTTGTAACATGTTTATGGGTTCAATCGCACAGCTTCTTCAACTGTTATCTTGATTGGCATT
GTCAGCACTCTGTCCCTGTGGGCCATCATGCCCTGTTGGTTCGTGTTCTATGGAGCTTATCTGTAT
TACCAGtgaacctacatactttttaaacgcaatgctatctacattcatgactacagatcgagaca
tggaaaactgagaccaaaaggaaactgattgtgtcatatctgtgtgtcataacctgatttttcc
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TGGCTGGGAATCCGTTTGGAAAGTTTGGGAGGTCTCATGGTTTGGTGGACTGCTTCATTAGCCGTC
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AACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAATTTGAGGATGTTGTTCTTCGTTAC
CGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGTTCTTGATTTCTCCAATGGATAAGGTGGGA
ATTGTTGGGAGGACAGGCGCTGGGAAATCAAGCCTCTTAAATGCCTTATTCAGGATTGTGGAGCTG
GAAAAAGGAAGGATTTTAATTGATGAATGCGACATTGGAAGATTTGGAAGTGTGGACCTACGTA
GTGGTCGGAATTATACCGCAAGCGCCAGTTCTTTCTCAGGTACCGTGAGATTCATCTTGACCCA
TTTAGTGAACACAACGACGCCGATCTCTGGGAATCTCTTGAGAGGGCACACTTGAAAGATACTATC
CGCAGAAATCCTCTTGGTCTTGATGCTGAGgtacttaattaataatttccatttgggaaagtctca
tgtattcagtaataataactcagtccttttgggtcagGTAAGTGAAGCAGGAGAGAATTTAGTGTT
GGACAGAGACAGTTGTTGAGTCTTGACAGTGCATTGTTACGAAGATCTAAGATACTTGTCTTGAT
GAAGCAACTGCTGCAGTTGACGTAAGAACTGATGTTCTCATCCAAAAGACCATCCGAGAAGAATTC
AAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTGACAAAGTTCTT
GTCCTTGATTCTGGAAAAGtacctatacaaaatattcgaccactacttgcacatatttaatacactt
ttgagactaacatatattgagattcccaacacctcagGTTACAGGAATTCAGTTCACCGGAGAATCTT
CTTTCAAATGGAGAAAGTTCTTTCTCGAAGATGGTTCAAAGTACAGGAAGTGAACGCGGAGTAC
TTACGTAGTATAACACTAGAGAACAAACGTACCAGAGAAGCTAACGGTGATGATTCAACACCTTTA
GAAGGTCAAAGGAAATGGCAAGCTTCTTCTCGTTGGGCTGCAGCTGCTCAATTTGCATTGGCTGTG
AGCCTCACTTCATCTCACACGACCTCCAAAGCCTTGAAATCGAAGATGATAACAGTATTTTGAAG
AAAACAAAGGACGCCGTCGTCACTTTACGCAGTGTCTTGAAGGGGAAACATGATAAAGAGATTGAA
GACTCTCTAAACCAAAGTGACATCTCTAGAGAGCGTTGGTGGCCATCTCTTACAAAATGGTCGAA
Ggtaacgttattcttaagatttctgatagcagatatagacataaagaattggtgaagtttcttgat
ctaataatttgtgtatatactctcagGGCTTGCCGTGATGAGCAGATTGGCGAGGAACAGAATGCA
ACACCCGATTACAATTTAGAAGGGAAATCGTTTGAAGGGGAAACATGATAAAGAGATTGAA
aggttacactaatagacctaaaactcccattttgatggaaacttttatttggattgcttgggatac
acgtaacaaaatgcccattaatcggtggtgaactatataggctatgcttcttttgggaaaaagaga
gtttgattacagaggatgtgatgataacacaattggaattcaaatttgcagcaaaatttgggagaa
aaaaaaagtcaatgagtgcaacatgcc

FIGURE 17C

SUBSTITUTE SHEET (RULE 26)

aacatgggtttcaacttctggacatggacaaccattggacataatttctctcacaggaccatgtttt
gtcattgacattttgcacaaaaatgttctattaacatatatctataaagaatttgaacaattgtt
aaaa aaacacttaaaatataaattgcaatacaaatttcctttttt

FIGURE 17D

SUBSTITUTE SHEET (RULE 26)

MGFEPLDWYCKPVPNGVWTKTVDYAFGAYTPCAIDSFVLGISHLVLLILCLYRLWLITKD
HKVDKFCRLRSKWFSYFLALLAAYATAEPLFRLVMRISVLDLDGAGFPPEAFMLVLEAFA
WGSALVMTVETKTYIHELWYVRFAYIYALVGDMLNVLVSVKEYYGSFKLYLYISEV
AVQVAFGTLLEFVYFPNLDYPGYTPVGTENSEDYEEELPGGENICPERHANLFDSEFFS
WLNPLMTLGSKRPLTEKDVWHLDTWDKTETLMRSFQKSWDKLEKPKPWLLRALNNSLGG
RFFWGGFWKIGNDCSQFVGPLLLNELLKSMQLNEPAWIGYIYAISIFVGVLGVLCEAQY
FQNVMRVGYRLRSALIAAVFRKSLRLTNEGRKKFQTGKITNMTTDAESLQQICQSLHTM
WSAPFRIIVALVLLYQQLGVASIIGALFLVLMFPIQTVIISKTKLTKEGQLQRTDKRIGL
MNEVLAAMDTVKCYAWENSFQSKVQTVRDELSWFRKAQLLSAFNMFILNSIPVLTVVS
FGVFSLLGGDLTPARAFTSLSLFSVLRFPLEMLPNIITQMVNANVSLNRLEEVLSTEERV
LLPNPPIEPGQPAISIRNGYFSWDSKADRPTLSNINLDIPLGSLVAVVGSTGEGKTSLSIS
AMLGELPARSDATVTLRGSVAYVPQVSWIFNATVRDNILFGAPFDQEKYERVIDVTALQH
DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVCILDEPLSALDAHVGQOVFE
KCIKRELQOTTRVLVTNQLHFLSQVDKILLVHEGTVKEEGTYEELCHSGPLFPRLMENAG
KVEDYSEENGEAEVHQTSVKPVENGANNLQKDGIEKNSKEGNSVLVKREERETGVVSW
KVLERYQNALGGAWVVMMLVICYVLTQVFRVSSITWLSEWTDSGTPKTHGPLFYNIYAL
LSFGQVSVTLINSYWLIMSSLYAAKKMHDAMLGSILRAPMVFFQTNPLGRIINRFAKDMG
DIDRTVAVFVNMFMGSIAQLLSTVILIGIVSTLSLWAIMPLLVFYGAYLYYQNTSREIK
RMDSTTRSPVYAQFGEALNGLSSIRAYKAYDRMAEINGRSMNDRFTLVNMAANRWLGI
RLEVLGGLMVWWTASLAVMONGKAANQAYASTMGLLLSYALSITSSLTAVLRLASLAEN
SLNSVERVGNYIEIPSEAPLVIENNRPPPGWPSSGSIKFEDVVLRYRPELPPVLHGVSFL
ISPMDKVGIVGRTGAGKSSLLNALFRIVELEKGRILIDECDIGRFGMLDLRKVVGIIPQA
PVLFSGTVRFNLDPFSEHNDAD...FSLERAHLKDTIRRNPGLDAEVTEAGENFSVGQRQ
LLSLARALLRRSKILVLDEATAAVDVRTDVLIQKTIREEFKSCTMLIIAHLNLTIIDCDK
VLVLDGSKVQEFSSPENLLSNGESSFSKMVQSTGTANAAYLRSITLENKRTREANGDDSQ
PLEGQRKWQASSRWAAAQFALAVSLTSSHNDLQSLIEDDNSILKKTDAVVTLSRVLE
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EM

FIGURE 18

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Fig. 19A

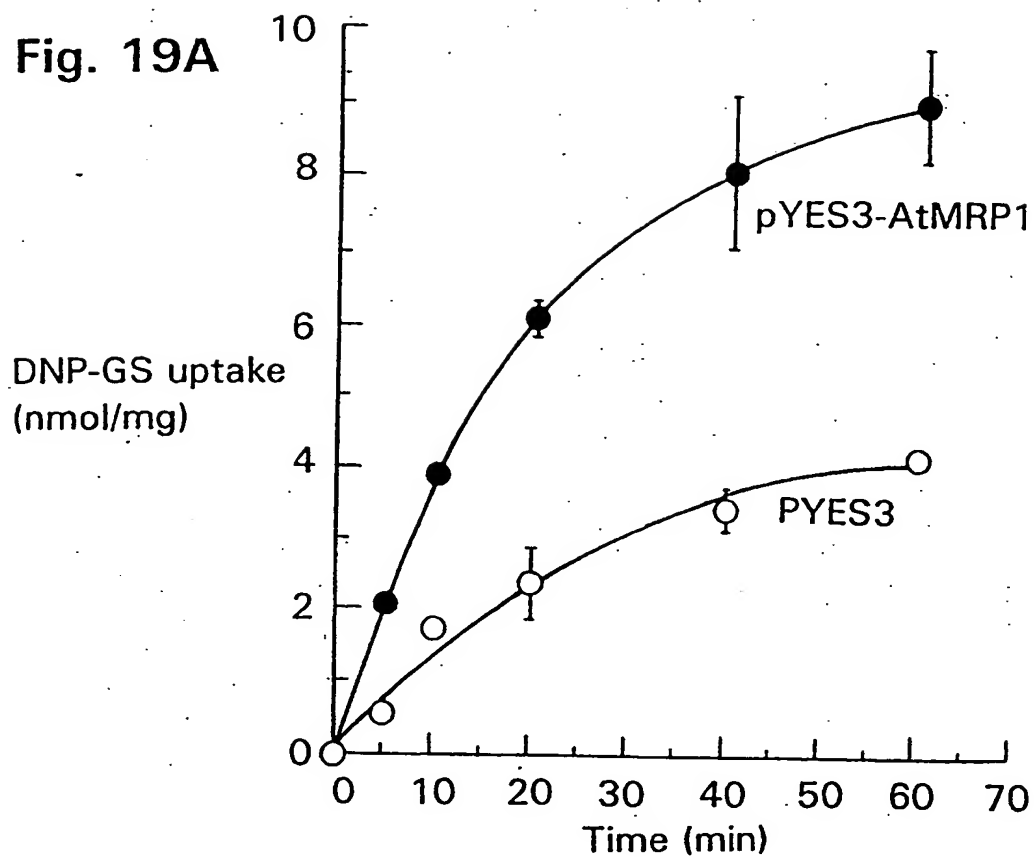
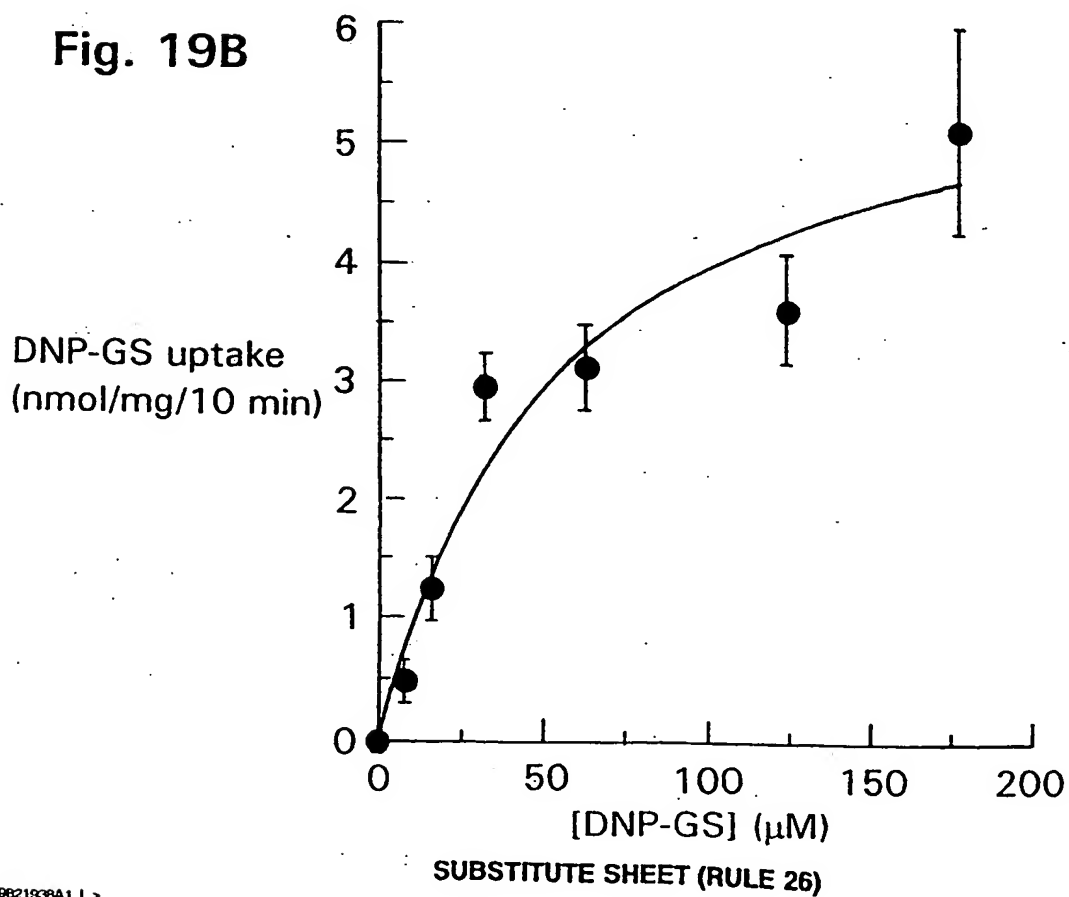


Fig. 19B



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Fig. 20A

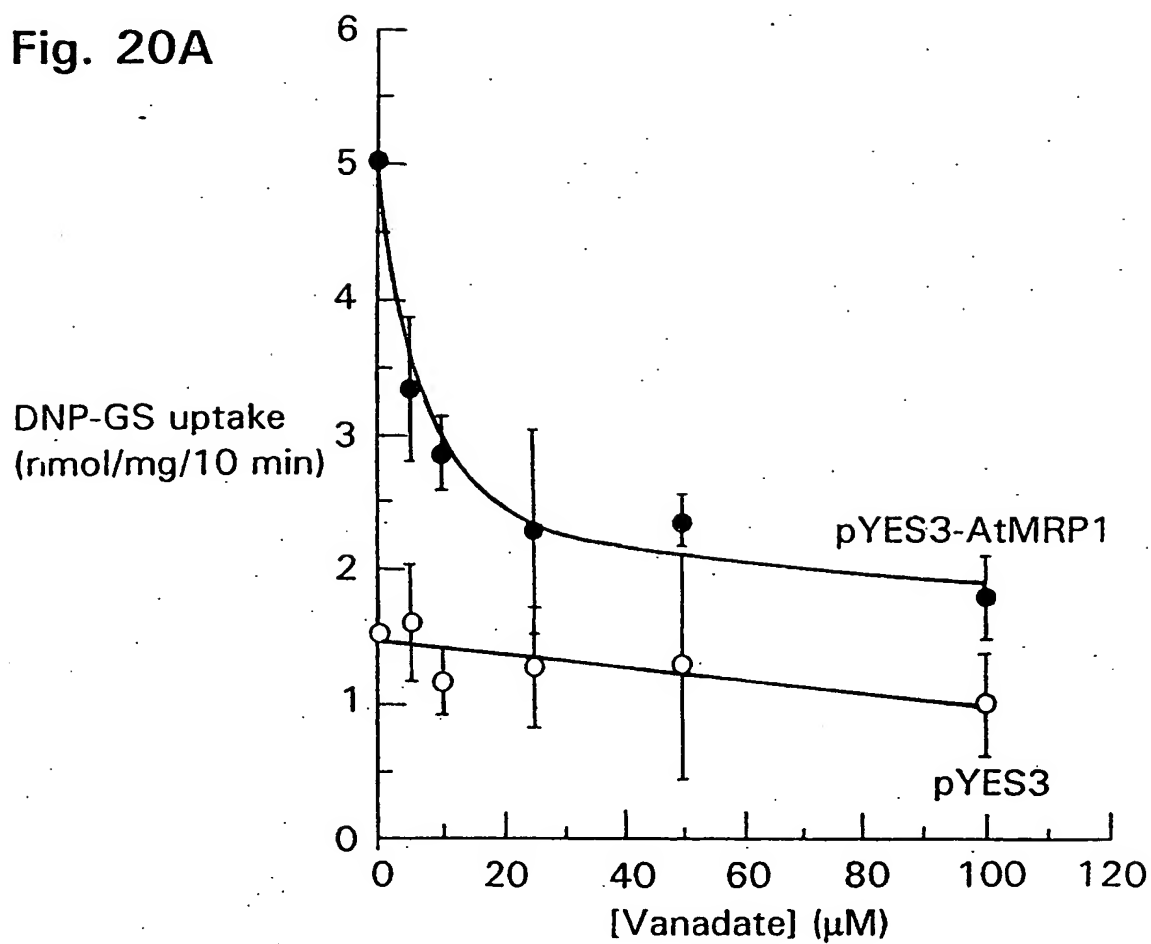
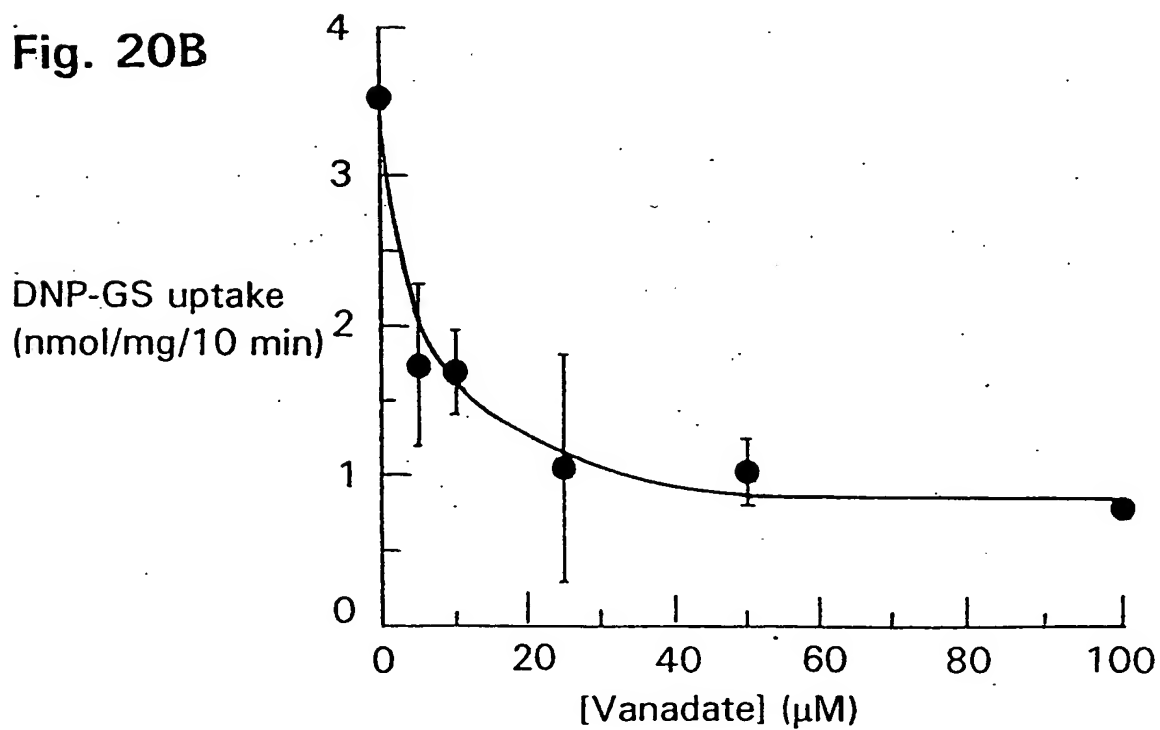


Fig. 20B



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Fig. 21

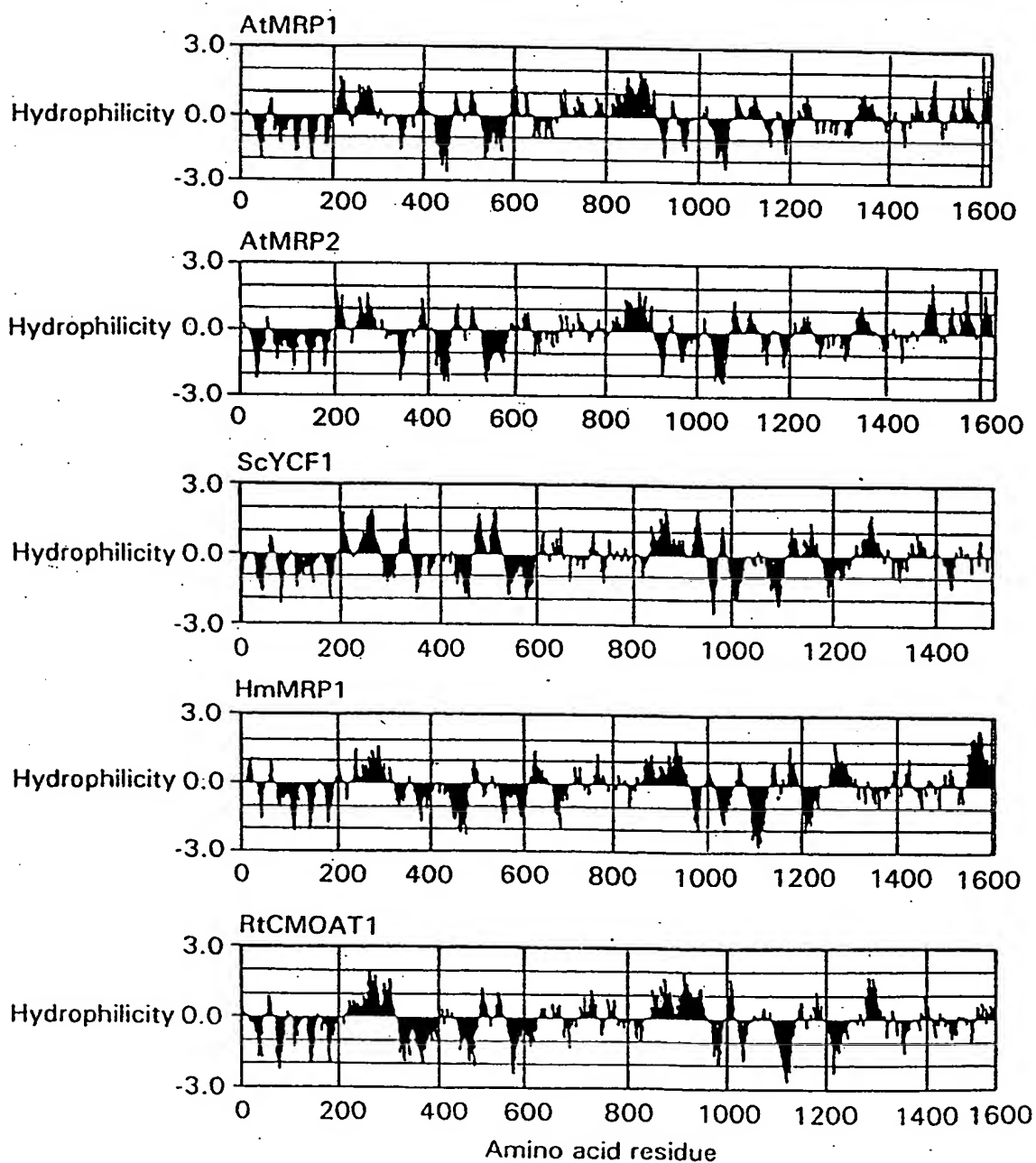
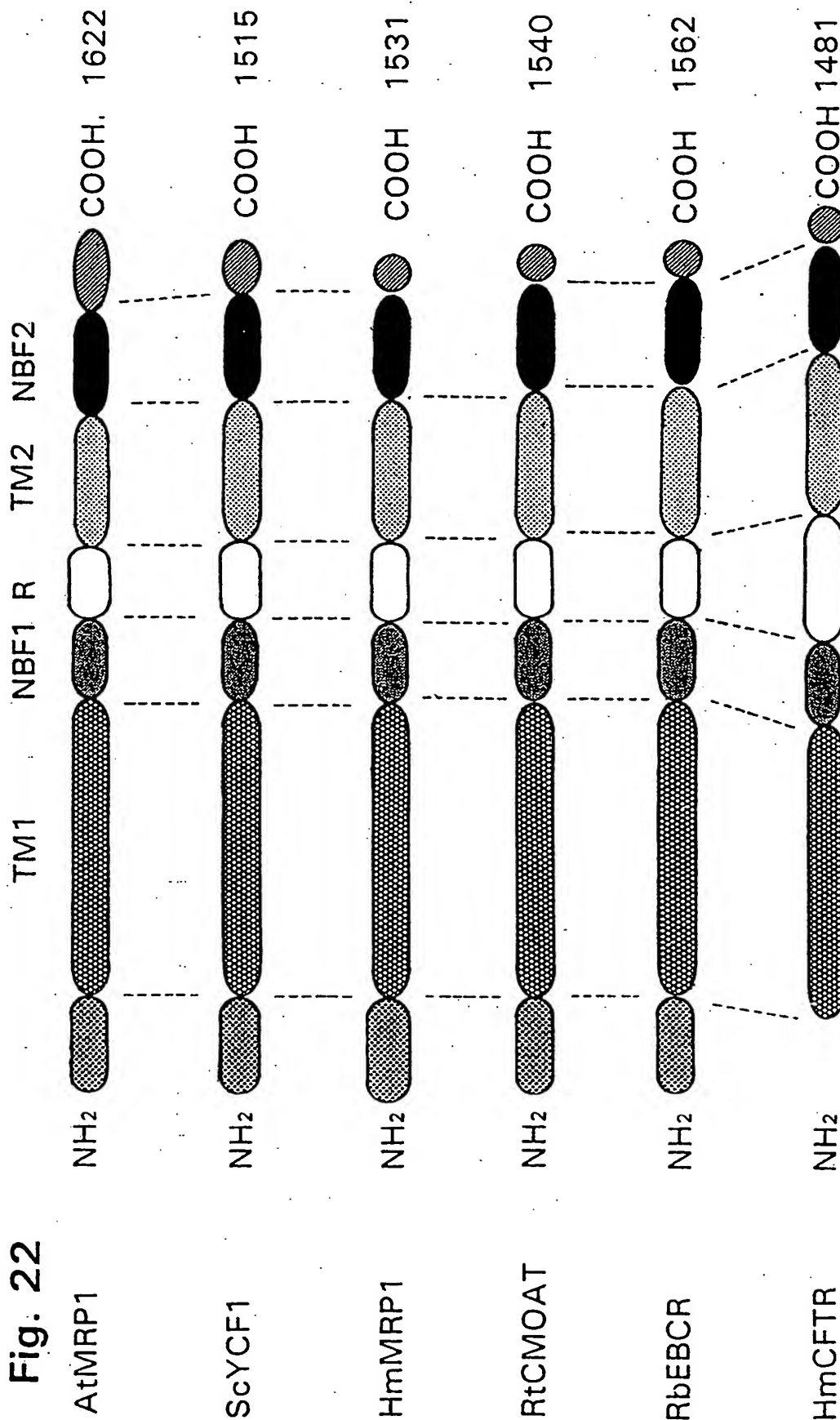


Fig. 22



AtMRP1 Promoter Sequence: 1253 bpContains:

- 2 Myb recognition sequences (atatcggtta and agttagtt).
- 1 xenobiotic regulatory element (GCGTG).
- 1 antioxidant response element (Gtgacaaa).

NB: Several "RNA instability determinants" (ATTTA) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

```
ttcacttttgtcctttttttcttaacatctacttttgtcatcagcaaattatctgtaaataa
gatagggtttatgcttattgctacaatgaacctaatcctatgatgtgtattgcaatttgcaa
ccatgcgagtttaattatttgtttactgctatagtgcattttatgatgtgtttttattaa
ttacaaaacagagcatcaaaaatcaaaagaacatcgcataatcgaaactatgctaataacct
ctcctcaatctttgttgttattatattcaagtagcttattctttgtttttattttacgatta
gatttctctagaATTTAATTTAatattATTTAatcatacttgatcaaggttttagcttaatc
aatatcggttatcggtgcatcctgcagattcaaatgatcaagtcataaatctacttatatgt
attatatatttagataccaccaacgaacaaaatcatattttctataacatttgtttgggtta
aatatATTTAaagatttgtaacagttgttcgggttcaaaactatcactttgtagttgtagga
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cattggatagtttaataaatgttaaaaaaataactaagtggtcaaagaagagatttgaaccata
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aaattacctatattttttattttctcaaaatatactaaaaattggatagagctattaaata
aaaaagatagaATTTAgagagaaatagcaacataatgaattataatataaatattttgtaaa
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gaacgttattgacttttcggcctctctctctctctatacaaatatggatcttcatttct
tcgtatagtgtgaagcagtgacgcacccATTTAatcatcatctccttataaatctcgaatctgc
cacagagagaGCGTGtgacaaaatgagttcataagattccgttatcgtcttctctgattcctc
caaatctccgg
```

FIGURE 23

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AtMRP2 Promoter Sequence: 1368 bpContains:

1 bZIP recognition sequence (cacgtg).
Xenobiotic regulatory element not found.
Antioxidant response element not found.

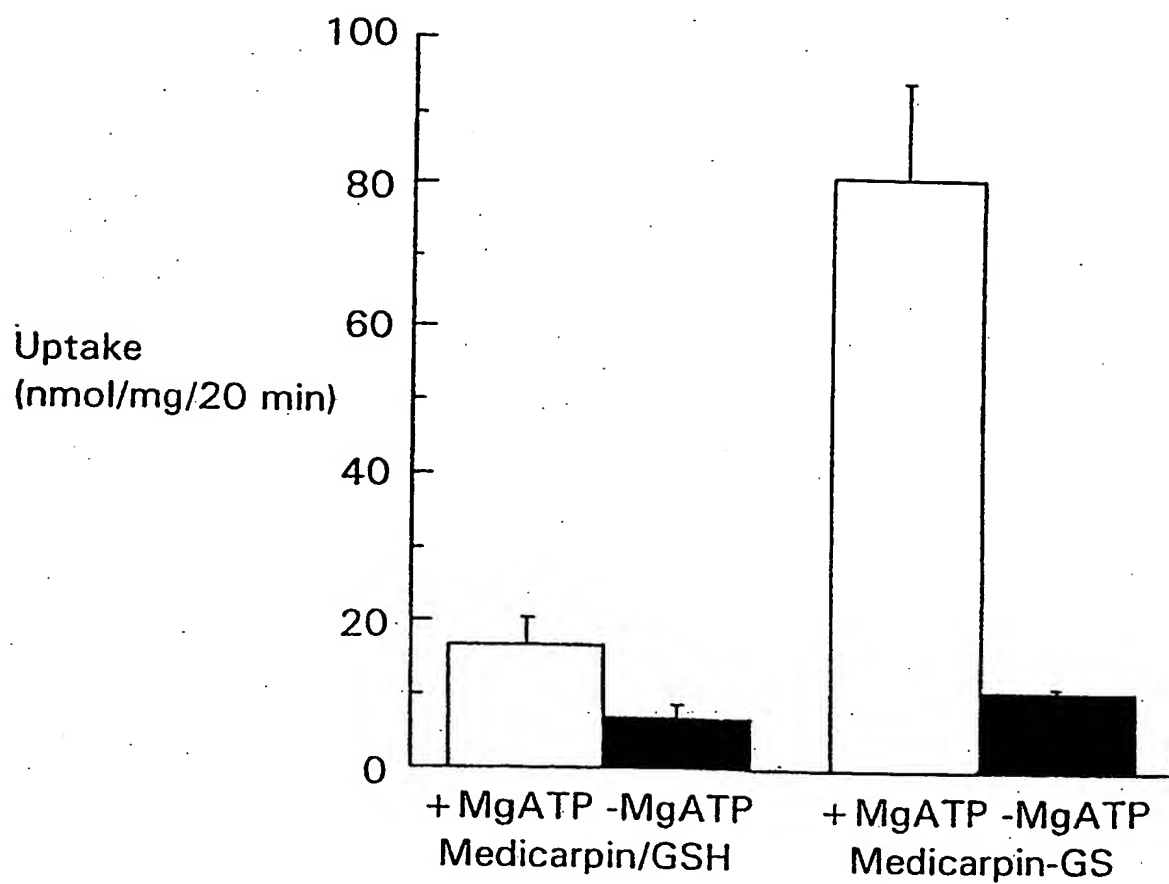
NB: Several "RNA instability determinants" (ATTTA) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

aaacaattggtgtatTTTTgaatttttcatgcaacgcacgtgaacagcttaattgcttgatt
ggaaacaaacctTTTTtagaattcattaatcagtttttaggtgTTTTggaaaattaacgaact
atagtgagagattaattaattttatattagtcTTTTtagtacacaaatcgaagtttcctag
atTTTTtcaaagttgaaaataatttgataatATTTATcaacaatgaatctacaaaaacat
aattTTTTtgccaaacaaataacaccgaaacaagattcattcactatTTTTgggttaaaaa
aaaaaatcaaaattacactattatgaagccaattTTTTgtatgcaaaaaacctgtatgtatc
aatttgTTTTgtattaaaaagtaagcATTTATgtctTTTTtataaataatagaaacactt
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ttttacgcgcgaatgtATTTATtatctttcgcactctgggtccatttcttgctacttgagc
acataatgattgATTTATgactttttaagttatgaaaATTTATtatttttggttgctatgg
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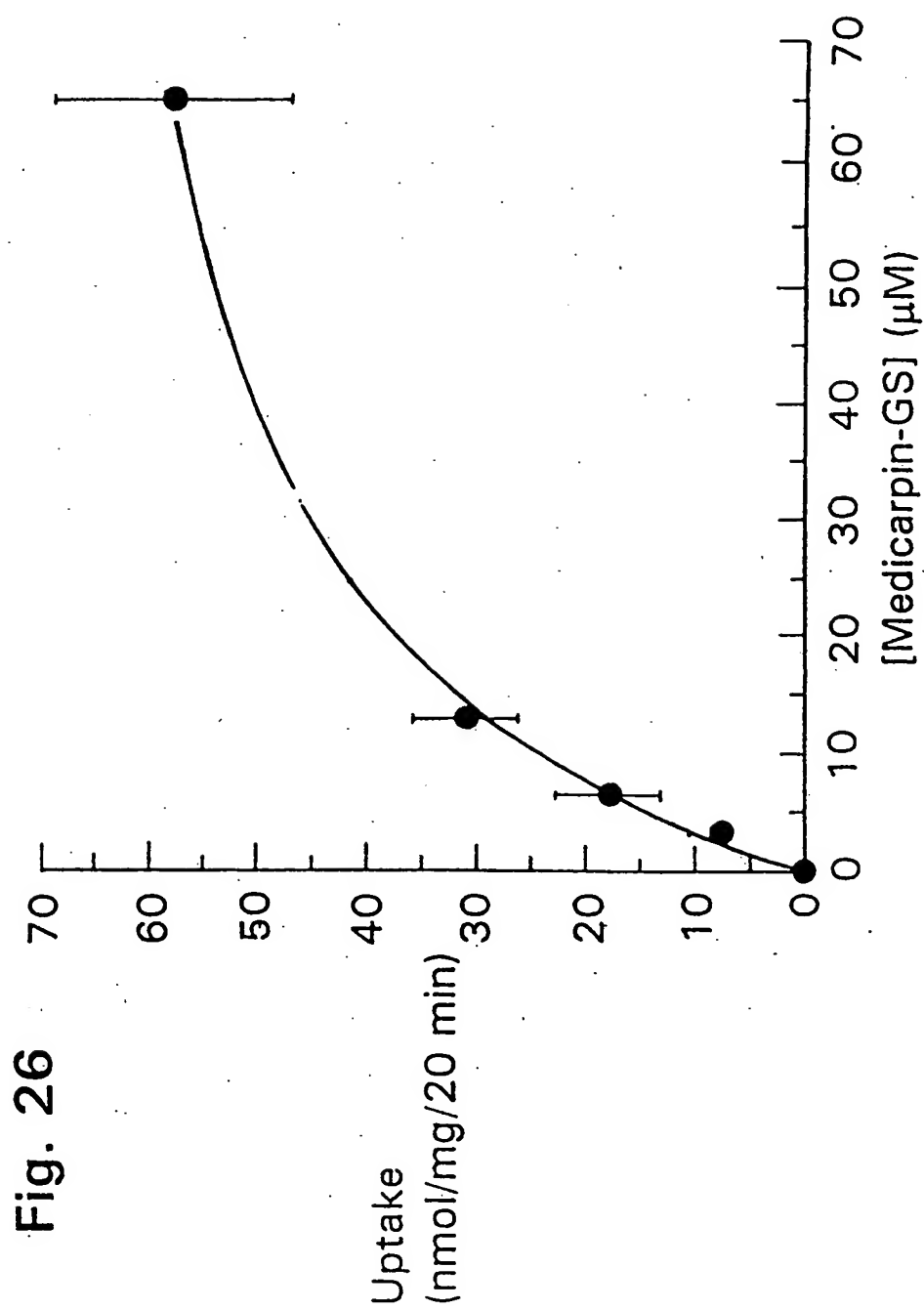
FIGURE 24

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Fig. 25



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Fig. 27A

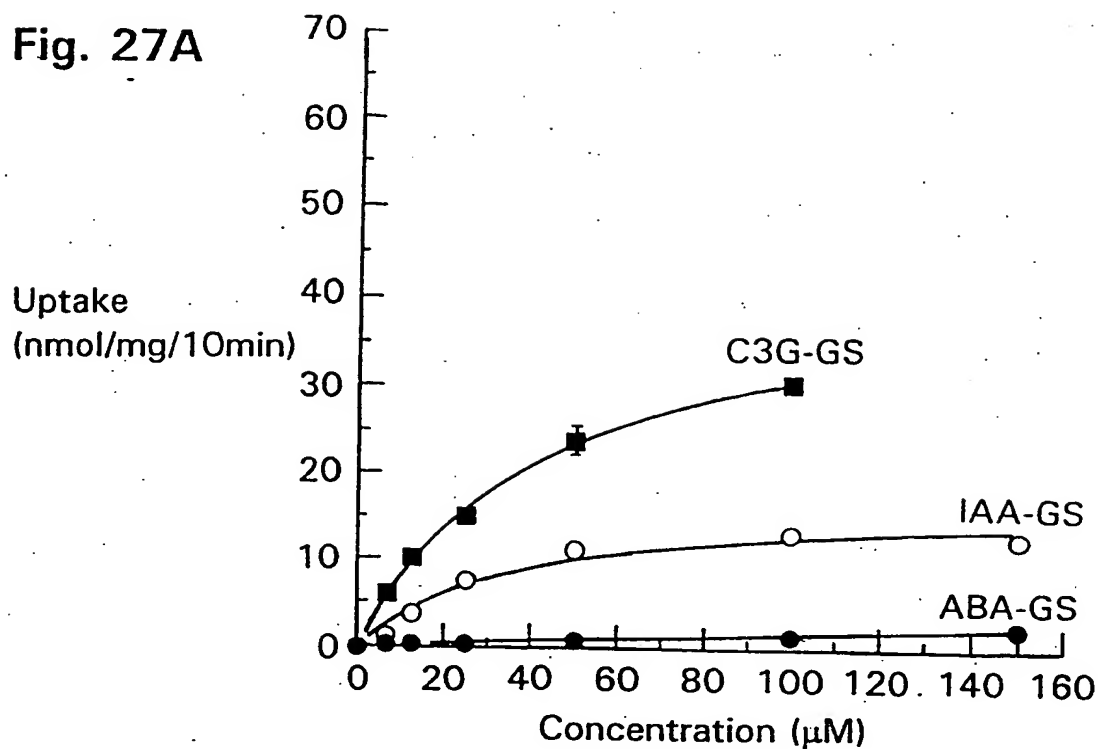
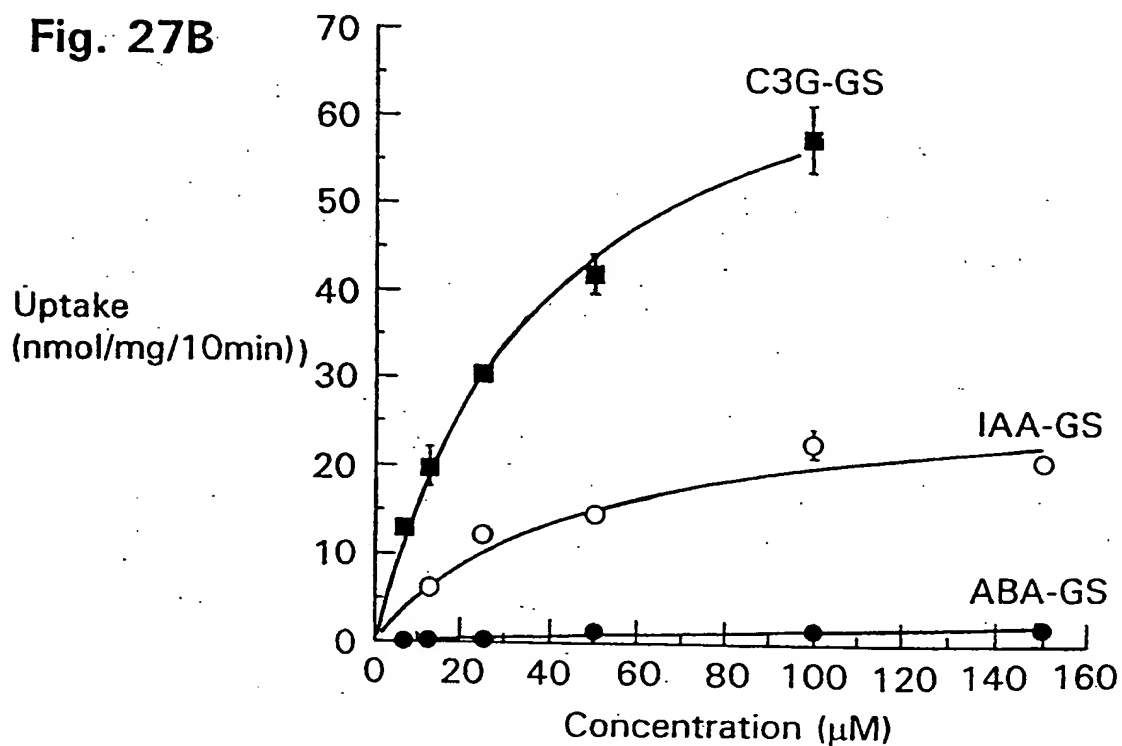


Fig. 27B



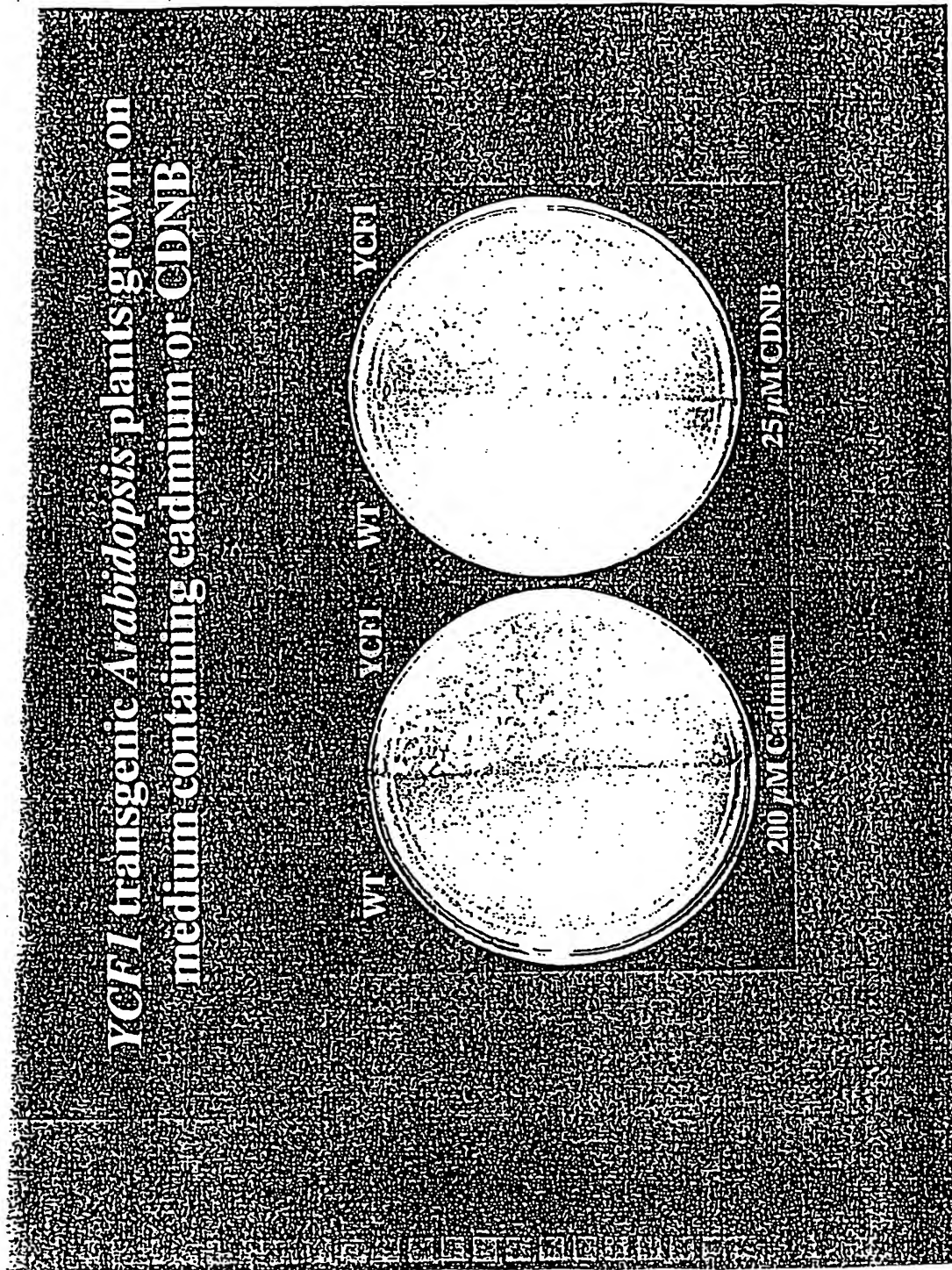


FIGURE 28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/21336

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG - Biotech files

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ISHIKAWA, T. et al. The GS-X Pump in Plant, Yeast, and Animal Cells: Structure, Function, and Gene Expression. Bioscience Reports. 1997. Vol 17, No. 2, pages 189-207, see entire document.	1-27
A	LI, Z.S. et al. Magnesium Adenosine 5'-Triphosphate-Energized Transport of Glutathione-S-Conjugates by Plant Vacuolar Membrane Vesicles. Plant Physiol., 1995, Vol 107, pages 1257-1268, see entire document.	1-27

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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* O* document referring to an oral disclosure, use, exhibition or other means	
* P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

26 JANUARY 1998

Date of mailing of the international search report

23 FEB 1998

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/21336

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	LI, Z.S. et al. A new pathway for vacuolar cadmium sequestration in <i>Saccharomyces cerevisiae</i> : YCF1-catalyzed transport of bis(glutathionato)cadmium. <i>Proc. Natl. Acad. Sci. USA</i> , January 1997, Vol 94, pages 42-47, see entire document.	1-27
T	LU, Y.P. et al. AtMRP1 gene of <i>Arabidopsis</i> encodes a glutathione S-conjugate pump: Isolation and functional definition of a plant ATP-binding cassette transporter gene. <i>Proc. Natl. Acad. Sci. USA</i> . July 1997, Vol 94, pages 8243-8248, see entire document.	1-27
A	MARTINOLA, E. et al. ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. <i>Nature</i> , 15 July 1993, Vol 364, pages 247-9249, see entire document.	1-27
A	LI, Z.S. et al. The Yeast Cadmium Factor Protein (YCF1) Is a Vacuolar Glutathione S-Conjugate Pump. <i>J. Biol. Chem.</i> , 15 March 1996, Vol 271, No. 11, pages 6509-6517, see entire document.	1-27

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/21336

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A01H 5/00; C07K 14/415, 16/16; C12N 1/13, 1/21, 5/10, 15/29, 15/64, 15/82

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205

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